de novo DNA METHYLTRANSFERASES IN ZEBRAFISH DEVELOPMENT

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by

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A Thesis Submitted in Partial Fulfilment of
the Requirements for the Degree of

Doctor of Philosophy

in the Department of Biochemistry

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Dean of Graduate Studies

Memorial University of Newfoundland

November 2009

Abstract

DNA methylation is a critical component of gene control, and is maintained by a family of of enzymes called the DNA methyltransferases. Methylation of CpG sequences promotes the formation of stably condensed, repressed chromatin. This process is important during development where DNA methylation is thought to have a role in lineage determination, X-chromosome inactivation, imprinting, and silencing of endogenous parasitic elements.

Our aim is to examine the role of methylation in gene silencing during vertebrate development using the zebrafish as a model. To this end we have identified and isolated a number of *de novo* DNA methyltransferase (*dnmt3*) gene sequences from the zebrafish (*Danio rerio*) and determined the relative expression levels of each during a variety of developmental stages. The zebrafish has six *de novo* DNA methyltransferase genes as opposed to the three found in mammals. In order to characterize the roles of the *dnmt* genes we have performed knockdown analysis by morpholino injection. In addition we have treated embryos with a methylation blocker, 5-aza-2'-deoxycytidine in order to examine effects of overall methylation disruption. Based on results from both of these approaches, we conclude that normal methylation is critical to notochord differentiation and development. Other processes where methylation may be relevant include heart, and central nervous system (CNS) development. Preliminary microarray experiments suggest a number of genes that are directly or indirectly regulated by methylation that may be pursued for further investigations of DNA methylation.

This work will not only allow us to use the zebrafish to examine the role of methylation in vertebrate development, but also develop it as a well-suited model for examining diseases related to abnormal methylation.

Acknowledgements

I would like to thank the numerous people who helped with this project. Foremost, I thank Ross McGowan for giving me the opportunity to work in his lab, for being such a talented supervisor, and for being a friend these past six years. I am particularly grateful for his care and patience in teaching, and for giving me freedom in my project but always accompanied with invaluable input and encouragement. In my future research years, I will do well to remember his unfailing sense of humour, and the importance of perseverance, and that Tom Waits and Swing make Fridays even better! Thanks to lab mates Beverly Morgan, Melanie Stenback, Cait Button, Mark Collins and Gordon Lake who have helped me move things forward through discussion, support, and technical assistance.

Thank you to my committee Dr. Hélène Volkoff and Dr. Brian Staveley. When first starting, Brian made me feel welcome, and I always appreciated his interest throughout my project. I am very grateful to Helene for her advice on a number of technical issues, and that her door was always open for a sit and discussion.

Many others assisted with this project through sharing of expertise and providing support. The many discussions held with Dr. Dawn Marshall as we hit the trails played a central role in helping me stay motivated and maintaining a fresh perspective, and as well always remembering to celebrate the successes. Thank you to fellow grad students Hope Brock, Corrine Wilkerson, Dr. Annika Haywood, and Semone Myrie for being supportive, Matt Nosworthy who was great for popping in for a discussion and for being

so generous with his time and resources in teaching me qPCR, and the rest of the Brunton/Bertolo lab that were always very helpful. Thanks to Donna Jackman for sharing her expertise in Western Blots, and Dr. Matthew Rise as well as The University Health Network Microarray Center, for their assistance with microarray experiments.

This project was made possible through the financial support of NSERC. Thank you also to Memorial University for awarding Graduate Student Fellowships and the Biochemistry Department for the Barrowman travel award.

More socially, I would like to say a warm thanks to the Feltham lunchroom crowd for providing stories and laughs everyday, especially in later years. Thanks to Betty Ann Lewis for organizing the biochemistry rowers, and for what it grew into.

And finally thank you to my friends outside the university and to my family. Thanks to Dr. John Sinclair for getting me started. Thank you to Gillian for her editing skills, and her always encouraging words. Thank you to my parents for all the cooked meals on visits, interest in my work, and unbelievable amounts of support. Without them I would not have taken this adventure. Thank you to my brothers and their families for time spent on the phone. Finally, thank you to Patrick for always helping, and for being so encouraging.

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Co-authorship Statement

Chapter 2 was published in BMC Developmental Biology. The cloning of the *dnmt3* genes depicted in Figure 2.2 was started by A.A Mhanni and C.C Dueck prior to my joining the project. I completed the cloning work and carried out the remainder of the experiments in this paper.

A version of chapter 3 will be submitted as an expression note. The bulk of this work was done by me with the exception of the of the brain, ovary, and muscle dissections carried out in order to obtain these tissues for the purpose of RNA extraction and expression analysis. I assisted Dr. Ross McGowan with these dissections.

List of Abbreviations

5azadC	5-aza-2'-deoxycytidine.
AP-1	Activator protein-1 transcription factor complex.
CH	Calponin homology domain.
DAB	3,3'-Diaminobenzidine
DNMT3	Human *de novo* DNA methyltransferase family (gene indicated by italics).
Dnmt3	Mouse *de novo* DNA methyltransferase family (gene indicated by italics).
dnmt3	Zebrafish *de novo* DNA methyltransferase family (gene indicated by italics). Also used to denote one of the six *de novo* methyltransferases in zebrafish (i.e. Zebrafish dnmt3 family is comprised of dnmt3, dnmt4, dnmt5, dnmt6, dnmt7 and dnmt8).
dop	Dopey gene.
ES cells	Embryonic stem cells.
flh	Floating head gene.
foxn4	Winged helix/forkhead transcription factor subclass N4.
gul	Gullivar gene.
hpf	Hours post fertilization.
ICF	Immunodeficiency, Centromeric Instability, and Facial Anomalies Syndrome.
kny	Knypek gene.
lev	Leviathan gene.
MBT	Mid-blastula transition.

MDS	Myelodysplastic syndrome.
MO	Morpholino.
mRNA	Messenger RNA.
ntl	No tail gene.
PCNA	Proliferating Cell Nuclear Antigen.
PHD	Plant homeodomain.
PWWP	Proline-Tryptophan-Tryptopan-Proline domain.
REST	Relative expression software tool.
RT-PCR	Reverse-transcription polymerase chain reaction.
SAM	S-adenosyl methionine.
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
sly	Sleepy gene.
sny	Sneezy gene.
tri	Trilobite gene.
tRNA	Transfer RNA.

Chapter 1: General Introduction

1.1 Epigenetics

The cells of our body differ from each other in order to make it possible to carry out a vast array of different functions. This is the very essence of multicellularity. However, with only a very few exceptions, each cell carries the full complement of DNA. The difference, therefore, from cell type to cell type in one individual is not a reflection of the DNA that is housed in the nuclei, but rather in the genes that are expressed. The control of gene expression at the transcript level in vertebrate cells may be broadly divided into three categories: 1) intrinsic promoter strength and availability of core transcription machinery, 2) the actions of promoter specific transcription factors, and 3) the control of DNA accessibility by altering chromatin structure (Cheng and Blummenthal 2008). The latter, and the focus of this thesis, is the least understood of these categories, and pertains to the study of epigenetics.

With entire genomes now sequenced, we are faced with the challenge of understanding how the genetic program is read (Bernstein *et al.* 2007). "Epi" means "on top of", so epigenetic literally means on top of the "genetic" layer. We are in the very early stages of understanding this superimposed layer of information, and even long held beliefs in this field are being challenged by recent findings (Metivier *et al.* 2008; Suzuki and Bird 2008). What can be stated, though, is that through regulation of chromatin structure and DNA accessibility, epigenetics modulate genome function (Bernstein *et al.* 2007). To give an idea of the complexities we face in understanding this area and its implications, something worth pondering is the fact that, though there is one genome in

an individual, there are as many epigenomes as there are types of cell (Suzuki and Bird 2008).

Defining epigenetics beyond its literal sense becomes more complicated. The journal *Nature* recently touched on the controversy surrounding the term "epigenetics," and what it means, in an article featuring the most disputed definitions in science (Pearson 2008). One unanimously agreed upon aspect in the field, however, is that DNA methylation classifies as an epigenetic phenomenon, satisfying as it does the classical definition of "the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence" (Riggs *et al.*, 1996 quoted in (Felsenfeld 2006) p.16).

In eukaryotic cells chromatin consists of DNA tightly associated with histone proteins. Some broader definitions of "epigenetics" allow for the inclusion of modifications that are made to such proteins (Bernstein *et al.* 2007). The functional unit of chromatin consists of approximately 146 base pairs of DNA wrapped around an octamer containing two copies of core histones, H2A, H2B, H3, and H4. In the recent past, histone proteins were simply thought of as spools onto which DNA was wound to allow compact packaging of hereditary material. It is becoming increasingly clear, however, that the marks laid down on the histone tails result in a much more extensive role for histones than simple spools, and in fact play an important signalling role in the cell (Sims *et al.* 2003).

One criticism of the classification of histone protein modifications as epigenetic is the lack of evidence for heritability of these marks. Nevertheless, the term "epigenome" may be loosely used to refer to cytosine methylation as well as the full repertoire of

histone modifications, with the understanding that only a subset of histone modifications may ever demonstrate epigenetic inheritance (Bernstein *et al.* 2007). And regardless of these nuances, what is clear is that the genomes of many eukaryotes carry these epigenetic marks and, interestingly, that they are not applied uniformly. Rather, they are laid down in patterns, and either signal and/or preserve local activity states, such as transcription or silencing (Suzuki and Bird 2008).

In eukaryotes, DNA methylation is the only known modification to actually occur on the DNA. In vertebrates this occurs almost exclusively at CpG dinucleotides (CpG denotes a cytosine that occurs next to guanine in the nucleotide sequence and these nucleotides are separated by a phosphate. This representation allows for distinction from CG base-pairing). Approximately 60%- 80% of all CpGs in the vertebrate genome are methylated (Chen and Li 2006; Bernstein *et al.* 2007). In contrast, there are more than 100 modifications including acetylation, methylation, phosphorylation, and ubiquitination that may be made to the histone proteins (Bernstein *et al.* 2007).

If we are to dissect and understand the biological significance of epigenetic phenomena, it is obviously critical to have a thorough understanding of not only the patterns that are laid down, but also how these patterns are established, maintained, and/or altered (Suzuki and Bird 2008). In this regard, a thorough knowledge of the enzymes that lay down these patterns is essential. There remain many questions surrounding what targets these enzymes, their control, their functional specificities as well as the overlap among them, their interactions, and the cross-talk that may occur between epigenetic layers. Only with these areas well in hand will we be properly equipped to address the physiological, developmental, evolutionary and biomedical significance of these

phenomena. With an appreciation for the various epigenomes of different cell types, and how they are laid down, we may distinguish normal state epigenomes from disease state epigenomes, understand how these transitions occur, and eventually be able to reverse, or even prevent these transitions.

1.2 DNA Methylation

DNA methylation is a suppressive mark. Though a deceptively simple modification, the addition of a methyl group to the 5-position of cytosine to give 5-methylcytosine (Figure 1.1) has a profound effect in a growing list of processes including tissue-specific gene expression (Oka *et al.* 2005), cell differentiation (Wu and Sun 2006), genomic imprinting (Smith *et al.* 2006), X-chromosome inactivation (Barr *et al.* 2007), developmental origins (Waterland and Jirtle 2004), genome defence and stability (Walsh *et al.* 1998), learning and memory (Levenson *et al.* 2006), carcinogenesis (Esteller *et al.* 2001), mental disorders (Mill *et al.* 2008), and aging (Casillas *et al.* 2003).

CpG methylation of promoter regions results in long term silencing of the associated gene through several mechanisms (Figure 1.2). In some instances, methylation may preclude recruitment of regulatory proteins by directly impeding the binding of transcription factors and thereby suppressing gene expression (Bird 2002). Alternatively, methylation effects may be mediated through the recruitment of regulatory proteins such as methyl-CpG-binding proteins, which in turn recruit histone deacetylases (Razin 1998). Histone deacetylation leads to the compaction of chromatin, rendering the chromatin inaccessible to transcriptional machinery.

Chromatin that is densely packed and said to be in a closed state refractive of expression is referred to as "heterochromatin", while chromatin that is relatively less compacted, said to be in an open state and at least potentially active, as it is accessible to transcriptional machinery, is referred to as euchromatin (Lewin 2002). Given DNA methylation and histone deacetylation lead to heterochromatin formation, and/or are involved in maintenance of this inactive state, heterochromatin is characterized by dense DNA methylation and deacetylated histones. Heterochromatin may be categorized as constitutive or facultative. In instances where the region of DNA is invariably heterochromatic, *i.e.* centromeric, pericentric, and telomeric regions, DNA methylation is referred to as constitutive. An example of facultative heterochromatin on the other hand is X-inactivation in mammals, where the inactive X-chromosome is heterochromatic, and the active X-chromosome, even though it has identical sequence, is part of euchromatin. Regardless of whether heterochromatin is constitutive or facultative, the chromatin is characterized by dense DNA methylation and deacetylated histones (Lewin 2002).

Although the majority of CpG sites are methylated across the mammalian genome, regions relatively free of methylation, referred to as CpG islands, are found in the promoter regions of, for example, approximately 60%-75% of human genes (Chen and Li 2006; Bernstein *et al.* 2007). A common suggestion in the literature, and one that has been challenged in more recent years, is that CpG islands are always unmethylated, with the exception of those in the inactivated X-chromosome, imprinted genes, and tumour suppressor loci in age related cancers. However, a growing list of genes with tissue-specific expression containing CpG islands have been shown to be methylated during development (Oka *et al.* 2005).

CpG islands may be defined as sequences greater than 200 bp which are enriched for CpG dinucleotides (G+C content greater than 50%). CpG islands are most commonly found in the promoter regions of housekeeping genes, though they are also present in approximately half of genes that have tissue-specific transcript expression (Goll and Bestor 2005).

1.3 The DNA Methyltransferases

The enzymes responsible for carrying out methylation of the cytosine base in CpG dinucleotides sequences are the cytosine-5 methyltransferases (Figure 1.3). Thus far, five mammalian DNA methyltransferases (Dnmts) have been identified (Meehan 2003). However, the activities of the enzymes may be broken into two main groups, maintenance methylation by DNA methyltransferase 1 (Dnmt1), and *de novo* methylation, performed mainly by the DNA methyltransferase 3 family (Dnmt3), also referred to as the *de novo* methyltransferases. The Dnmt3 family is comprised of two catalytically active enzymes, Dnmt3a and Dnmt3b, while the third member, Dnmt3l, is catalytically inactive on its own, yet acts as an essential co-factor (Robertson 2001). Dnmt2, the most widely conserved of the methyltransferase families (Meehan 2003), is likely to have a role as a tRNA methyltransferase, and investigations of its function as a DNA methyltransferase are still ongoing (Rai *et al.* 2007).

The defining aspect of cytosine-5 methyltransferases is the presence of up to 10 conserved motifs in the C-terminal catalytic domain, and a number of other common motifs (Figure 1.3) (Chen and Riggs 2005). Summaries of the current state of knowledge

for the maintenance methyltransferase, as well as Dnmt3a and Dnmt3b, are provided below. Critical areas requiring closer examination for a more complete understanding of DNA methylation, particularly with its relevance to development, are highlighted.

a) Dnmt1

One of the features of DNA methylation is that the mark is stable and heritable through cell division (Figure 1.4). Therefore, CpG dinucleotides that are converted to the hemimethylated state during DNA replication must be faithfully replicated to the daughter DNA strand in order to prevent passive loss of these marks. Dnmt1 is considered a maintenance methyltransferase based on both *in vitro* and *in vivo* evidence. Dnmt1 shows a 5-40 fold preference for hemimethylated vs. unmethylated DNA *in vitro* (Turek-Plewa and Jagodzinski 2005). Expression of Dnmt1 at the transcript level is consistent with its role as a maintenance methyltransferase as it is highly expressed in proliferating cells and is ubiquitously expressed in somatic cells (Brenner and Fuks 2006). Additional support for the function of Dnmt1 as a maintenance methyltransferase comes from *in vivo* studies showing localization of Dnmt1 to DNA replication foci during S phase and interaction with the DNA polymerase processivity factor proliferating cell nuclear antigen (PCNA). As well, inactivation of Dnmt1 results in widespread demethylation of examined sequences, but does not affect *de novo* methylation of newly integrated retroviral DNA (Chen and Li 2006).

Several areas of research are ongoing to investigate the role(s) of Dnmt1 in development. Particular interest is centered on its role in maintaining imprinted genes through a wave of demethylation during mammalian development (Dean 2008), its interactions with other epigenetic layers (Estève *et al.* 2006), and a study in zebrafish has

provided evidence for dnmt1 as having a role in terminal differentiation in a number of vertebrate tissues (Rai *et al.* 2006).

b) *Dnmt3* Family

The Dnmt3 family is of significant interest as these enzymes establish the methylation patterns during gametogenesis and early embryogenesis (Figure 1.4). There are only two catalytically active members in this family: Dnmt3a and Dnmt3b. Despite Dnmt3l's similarity to Dnmt3a and Dnmt3b, it lacks some of the critical catalytic motifs, rendering it inactive.

Expression patterns of *Dnmt3a* and *Dnmt3b* are consistent with their proposed role as *de novo* methylating enzymes. For instance, both are highly expressed in embryonic stem (ES) cells (Okano *et al.* 1998), early embryos (Watanabe *et al.* 2002), and developing germ cells (Lees-Murdock *et al.* 2005), - all instances where *de novo* methylation takes place. On the other hand, they are downregulated in somatic tissues of postnatal animals, where one can expect fewer requirements for *de novo* methylation (Okano *et al.* 1999). Additional support for these being *de novo* methyltransferases is given by the fact that *de novo* methylation is disrupted when *Dnmt3a* and *Dnmt3b* are inactivated by gene targeting in ES cells as well as in early embryos (Okano *et al.* 1999). Additionally, *de novo* methylation results when *Dnmt3a* and *Dnmt3b* are over-expressed in mammalian cells or transgenic flies (Lyko *et al.* 1999). More recently, Dnmt3a and Dnm3l have been shown to be essential for the establishment of methylation imprints during gametogenesis (Kaneda *et al.* 2004). Although Dnmt3l is catalytically inactive, it likely has a role in targeting of Dnmt3a to particular genomic regions. Collectively, these

findings provide strong evidence for Dnmt3a and Dnmt3b as *de novo* methylating enzymes that establish DNA methylation patterns during embryogenesis and gametogenesis.

There is evidence, however, that both Dnmt3a and Dnmt3b may also have a role in maintaining methylation levels along with dnmt1, and there is support for interaction among all of these proteins. These interactions and their significance are not well understood (Chen and Li 2006).

The critical nature of *de novo* methylation in development has probably been most dramatically demonstrated in studies employing knockout techniques in mice where *Dnmt3a* null mice are undersized and die within 4 weeks of birth, and *Dnmt3b* null mice do not even develop to term (Okano *et al.* 1999; Meehan 2003). Although *Dnmt3l* deficient females are normal, the embryos derived from those females do not develop to term, and are lacking maternal-specific imprints (Kaneda *et al.* 2004). All of the above demonstrate very clearly that the Dnmt enzymes play a very fundamental and critical role in normal and abnormal mouse development.

We may gain some insight into the role of the *de novo* dnmts and their targeting from their structure (Figure 1.3). Dnmts generally are comprised of two domains: a highly conserved catalytic domain in the C-terminal region, and a more variable regulatory domain in the N-terminal domain (Brenner and Fuks 2006). Both Dnmt3a and Dnmt3b have in their N-terminal regulatory domains a variable region, a PWWP (Proline-Tryptophan-Tryptophan-Proline) domain, and a cysteine-rich region PHD (Plant homeodomain) (Chen and Li 2006). The catalytic domain, on the other hand, contains up to ten characteristic motifs, of which six are highly conserved, carrying out various steps

including binding cytosine and the methyl-donor S-adenosyl methionine (SAM) (Turek-Plewa and Jagodzinski 2005).

The PWWP domain, which consists of 100-150 amino acids containing a highly conserved PWWP motif, is likely to have a functional significance in targeting the Dnmt3s to pericentric heterochromatin. The significance of this motif has been revealed by the human disorder Immunodeficiency, Centromeric Instability, and Facial Anomalies (ICF) syndrome. Although the majority of mutations in DNMT3B causing ICF are located in the C-terminal catalytic domain, in a couple of instances missense mutations were shown to occur in the DNMT3B PWWP domain. When analogous mutations in mouse cells were examined, a lack of constitutive pericentric heterochromatin targeting was found (Ehrlich *et al.* 2008).

People suffering from ICF have characteristic chromosomal abnormalities such as chromatin decondensation, rearrangements, and DNA hypomethylation, specifically in the pericentric chromatin of chromosomes 1, 9, and 16. Patients have high mortality in childhood due to immunodeficiency, and also usually display facial anomalies such as broad flat nasal bridge, widely spaced eyes, and low set ears (Ehrlich *et al.* 2008). Mental retardation has been observed in about one-third of the patients with ICF (Ehrlich *et al.* 2008).

The conserved Plant Homeodomain is present in all three members of the dnmt3 family. This domain is involved in protein-protein interactions. Examples discovered to date include interactions with transcription factors Myc and RP58, as well as interactions with heterochromatin protein HP1, histone deacetylases (HDACs), and the histone methyltransferase Suv39h1 (Chen and Li 2006). These interactions are particularly

interesting as they provide mechanistic links that could explain some of the already indicated cross-talk between epigenetic layers; again, however, much research is needed before an understanding of the functional significance of these interactions will be achieved.

Splice variants and promoter specific variants have been identified for both the *Dnmt3a* and *Dnmt3b* genes in mice and humans (Figure 1.5) (Meehan, 2003). In fact, there are two variants of Dnmt3a, and six Dnmt3b isoforms (Hermann *et al.* 2004). An emerging consideration is that the number of isoforms (Meehan, 2003), and the differences in their timing and levels of expression is a source of functional diversity for the *Dnmt3* genes (Li, 2002). Discerning the role of each gene and isoform will no doubt provide great insights into how methylation patterns are established (Li, 2002).

The difference in localization between Dnmt3a and Dnmt3a2, a Dnmt3a isoform that lacks the variable region, highlights the diversity that may result depending on presence or absence of particular motifs. Dnmt3a2 displays a diffuse nuclear localization pattern, while Dnmt3a is targeted to heterochromatin regions (Chen and Li 2006). This not only suggests the variable region of Dnmt3a is involved in heterochromatin targeting, but also implicates Dnmt3a2 as a gene-specific DNA methyltransferase since it is diffusely localized. Clearly, discovering which genes are being targeted, and the timing of these events will provide critical insight into the developmental program.

Despite some knowledge concerning a number of the dnmt structural features, there is still much to be learned as to the specific roles each of the methyltransferases has in establishing methylation patterns and how they are regulated. Given that dnmts do not appear to have any sequence specificity beyond CpG dinucleotides, the question also

remains as to what specific interactions allow these enzymes to target specific sequences. That this challenge is being met is illustrated by the significant interest shown in the interacting proteins of the dnmts and, indeed, a handful of these interacting proteins have been identified, some of which are mentioned in the above paragraphs, which are involved in regulation of chromatin structure and gene expression (Chen and Li 2006).

1.4 Mechanism of Methylation

The mechanism of DNA methylation involves binding of the methyltransferase to the DNA, and in a process referred to as "base flipping" the cytosine is everted so that it projects out of the double helix (Figure 1.6) (Voet and Voet 2004). This is followed by the covalent attack of a conserved Cys nucleophile on cytosine carbon 6, a methyl group is transferred from the methyl donor SAM to the activated cytosine carbon 5, and this is then followed by the release steps (Cheng and Blummenthal 2008). This unusual mechanism is necessary to create an unstable intermediate in order to methylate the relatively unreactive fifth position on cytosine in the DNA.

1.5 Methylation Dynamics in Vertebrate Development

DNA methylation is required for normal development in mammals, and likely all vertebrates. Dynamic methylation changes are seen during development, involving whole genome demethylation and *de novo* methylation (Figure 1.7) (Reik *et al.* 2001). Both sperm and ova DNA have been demonstrated to be highly methylated in mammals, however, upon fertilization, the paternal genome undergoes rapid, active demethylation.

While the maternal genome also undergoes a wave of demethylation, in contrast to the active demethylation of the paternal genome, demethylation here is in keeping with a passive process, where maintenance methylation is not copied onto the daughter strand following replication (Dean 2008). This reprogramming of gamete DNA occurs genome-wide, with the exception of imprinted genes in mammals that maintain their methylation status throughout this process. Following implantation, a wave of *de novo* methylation occurs, establishing a new embryonic methylation pattern that is maintained in the adult (Dean 2008). These events are believed to be critical in establishing patterns of gene expression and consequently driving cell lineage determination (Li 2002).

1.6 Zebrafish as a Model for Development

The zebrafish was selected as a developmental model during the 1980's by George Streisinger at the University of Oregon based on several important attributes and since then, these small freshwater teleosts have attained a prominent place in vertebrate development research. A number of the traits that bring this model to the forefront of development research are the very ones we have used here to gain understanding of the DNA methyltransferases in development.

Practically, zebrafish are favourable for their ease in care and space requirements (Figure 1.8), and the fact that, when kept on a specific light cycle (14 hours light, 10 hours dark), they breed within the first hour of the light cycle -what would be dawn in the wild. Zebrafish have a high fecundity and only a three-month generation time (Figure 1.8). Most importantly, however, external fertilization and optically clear embryos

greatly facilitate observation of development, including both normal development and potentially subtle abnormalities following experimental manipulation (Figure 1.9) (Grunwald and Eisen 2002).

Rapid ontogeny is another practical attribute of zebrafish for developmental studies (Link and Megason 2008). Following fertilization, cytoplasmic streaming begins, whereby a large blastodisc is formed on top of the yolk (Figure 1.9A). Synchronous cell divisions occur for the next two and three-quarter to three hours (when embryos develop at 28.5 °C). At 2.75 hpf (hours post fertilization), the blastodisc is at the 512-cell stage; until this point cell divisions are synchronous, and no cell growth takes place giving rise to an increasing number of cells of decreased size (Schier and Talbot 2005). This division without growth leads to a critical threshold of DNA:cytoplasm ratio for cells, and division becomes asynchronous during the mid-blastula transition (MBT, panel j). This marks the activation of the zygotic genome which will now govern development, though some zygotic genes do become active prior to this point (Schier and Talbot 2005).

At approximately 5 hpf, zebrafish blastulas enter gastrulation (Figure 1.9A). By the process of epiboly, the blastoderm thins and spreads over the yolk, until, by the end of gastrulation, the blastoderm completely covers the yolk (Link and Megason 2008). By 6 hpf, a thickening at the dorsal blastoderm margin (Figure 1.9A panels q and r indicate margin and shield) is apparent as a result of dorsal accumulation of cells by convergence and extension. This thickening, referred to as the shield, is the analogous structure to the node of mammals or Spemann-Mangold organizer of amphibians (Webb and Miller 2007). By 10 hpf somitogenesis has begun, and by 24 hpf the major subdivisions of the nervous system are in place. As well, rudiments for the primary organs are in place and

at this point in zebrafish development, and the heart begins beating. Organogenesis is complete by 72 hpf, and embryos are capable of swimming. For the first 5 days of development, nutrition is provided to the embryo by the yolk, however, at 5 days post-fertilization, larval zebrafish begin foraging and feed externally (Link and Megason 2008). Although pigmentation begins at 24 hours of development, later stages can be rendered transparent if desired with the use of melanin synthesis inhibitors (Link and Megason 2008). The molecular events that orchestrate these processes of morphogenesis and organogenesis are largely conserved to processes in humans, making zebrafish an excellent model for the study of fundamental processes of development, disease modeling, and drug evaluation (Chen and Ekker 2004).

1.7 The Zebrafish Genome

The zebrafish genome is diploid, and its estimated size is 1600 Megabases (Mb) (Link and Megason 2008). There are 25 paired chromosomes which have been assembled by the Sanger Center (http://www.sanger.ac.uk/Projects/D_rerio/). The conserved synteny that is observed between zebrafish and humans is actually even greater than between humans and mouse, which have undergone extensive chromosome rearrangements (Dahm 2002). One trait that is important to be mindful of when analysing gene function in this model, however, is that during teleost evolution the genome underwent a genome duplication followed by massive gene loss that has resulted in duplicate gene representation of approximately 30% of genes in zebrafish compared to mammalian orthologs (Postlewait *et al.,* 1998). This event is estimated to have occurred

approximately 450 million years ago. Based on studies to date, the general rule appears to be that the divergence of duplicated genes in zebrafish has been most observed in the control regions and not as much in functional divergence, though certainly there are likely some exceptions (Link and Megason 2008).

1.8 Zebrafish and Epigenetics

Several lines of evidence are emerging which indicate DNA methylation, and epigenetics in general, play a critical role in zebrafish development, as they do in mammals. Zebrafish have an identified *dnmt1* (Mhanni *et al.* 2001), *dnmt2* (Rai *et al.* 2007), and six *de novo* methyltransferases that are homologous to mammalian *Dnmt3a* and *Dnmt3b* (Shimoda *et al.* 2005; Smith *et al.* 2005). Zebrafish do not have an identified *dnmt3l*, and do not imprint genes in development. With the presence of the *de novo* DNA methyltransferases, and the many genes implicated with the histone code, we may first characterize this system, but more interestingly, we may delve into explorations of function of epigenetics in development and beyond.

One of the major pieces of evidence that DNA methylation has a role in zebrafish development comes from examining genomic methylation changes during development (Mhanni and McGowan 2004; Mackay *et al.* 2007). Interestingly, zebrafish genomewide methylation levels are dynamic during development, and show similar trends to what is observed in mammals. Sperm is heavily methylated, while embryonic genome methylation levels are relatively hypomethylated through cleavage and early blastula stages. Genome methylation levels increase in late blastula stages, and show relatively

heavy methylation levels by gastrulation (Mhanni and McGowan 2004; Mackay *et al.* 2007).

1.9 Principal Hypothesis and Overall Objectives

The **principal hypothesis** is that DNA methyltransferases function both together and independently with different factors to target and repress specific genes and constitutive heterochromatin that are necessarily silenced for development and cellular differentiation. The **overall objectives** are to (1) establish the zebrafish as a model for investigations of *de novo* DNA methylation, (2) determine the relevance of *de novo* DNA methyltransferases for different stages of normal development, and (3) present a precise compilation of the genes regulated by DNA methylation, providing insight into central developmental processes.

1.10 Significance

In combining the strengths of the zebrafish model with techniques capable of examining unexplored aspects of the *de novo* methyltransferases, this work provides new insight into the roles the dnmts play in controlling progression of the developing embryo through the various stages of development. Evidence suggests roles ranging from stages immediately following fertilization to terminal differentiation; by directly examining these processes in developing embryos, we have gained a more complete perspective for specific and overlapping contributions of the different dnmts. Characterizing the enzymes responsible for this epigenetic mechanism of gene control, as well as providing

genes that are either directly or indirectly regulated by methylation during development in zebrafish, a model rapidly gaining attention not only for normal development studies, but also for disease modeling and drug discovery (Langheinrich 2003), is highly relevant given the potentially reversible nature of aberrant epigenetic marks, or epimutations (Brueckner and Lyko 2004).

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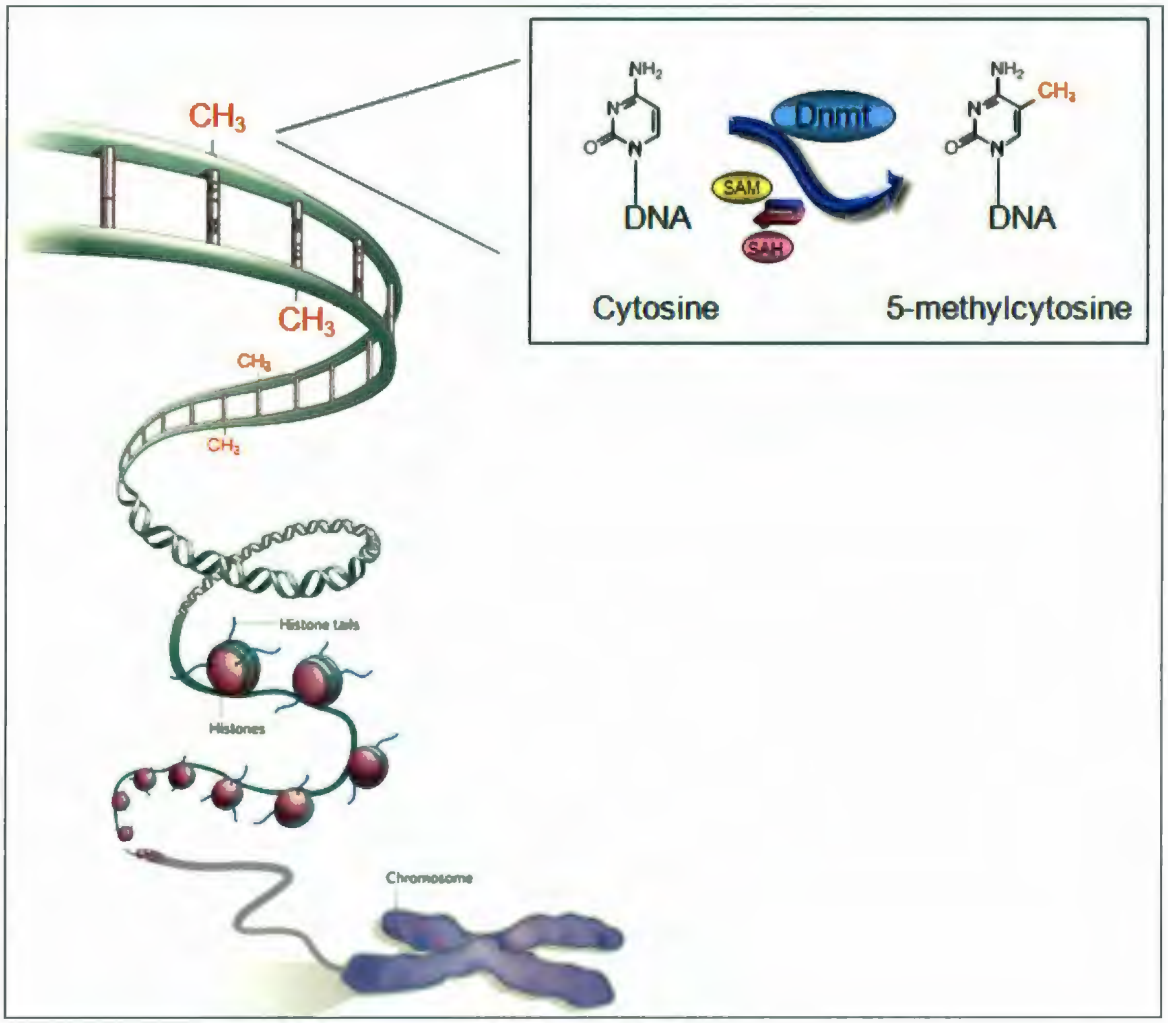


Figure 1.1 Methylation of cytosine to 5-methylcytosine. Methylation in vertebrates typically takes place at CpG dinucleotides. Methyltransferase enzymes transfer a methyl group to cytosine from the methyl donor S-adenosyl-L-methionine (AdoMet or SAM) giving rise to 5-methylcytosine and S-adenosyl-L-homocysteine (SAH) (adapted from Qui, 2007).

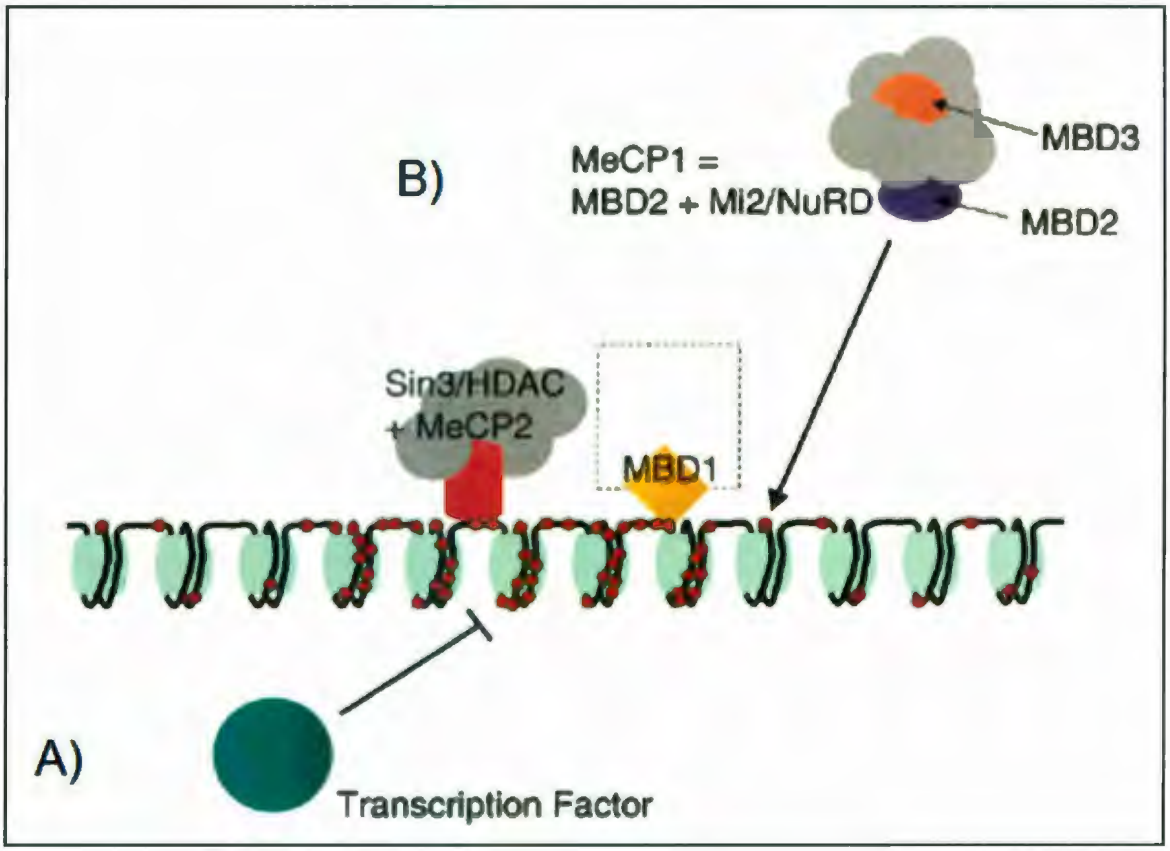


Figure 1.2 Mechanisms of gene silencing by DNA methylation. DNA (black line) is coiled around histone proteins (light blue). Red circles represent methylated CpG sites. A) Methylation may prevent binding of a transcription factor when the methylated CpG lies within a recognition site. B) CpG methylation may recruit protein complexes (above the line) such as MeCP2 or MeCP1, that lead to the compaction of chromatin via modifications made to the histone tails, such as histone deacetylation, rendering the chromatin inaccessible to transcription machinery (adapted from Bird 2002) (MeCP = methyl CpG binding protein; MBD = methyl binding domain; HDAC = histone deacetylase).

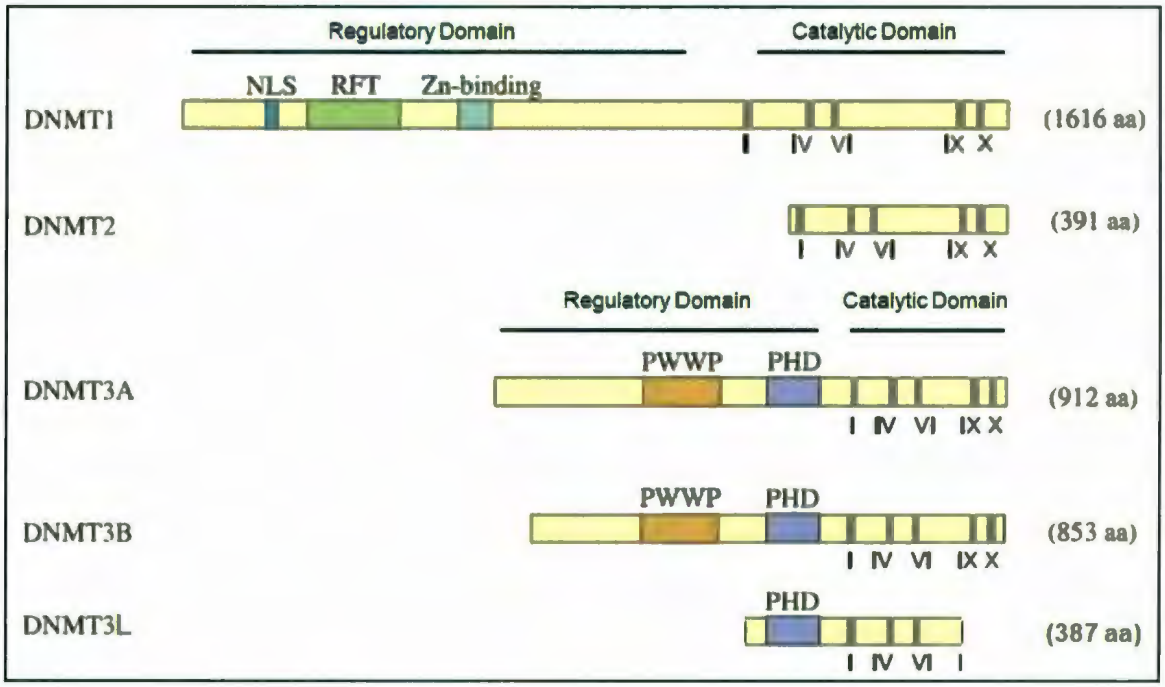


Figure 1.3 Schematic of the human DNA methyltransferases. Three families are known; DNMT1, DNMT2, and DNMT3. These proteins, with the exception of DNMT2, possess an N-terminal regulatory domain and a C-terminal catalytic domain. DNA methyltransferases contain up to ten conserved motifs which are indicated by Roman numerals. NLS, nuclear localization signal; RFT, replication foci-targeting domain; PWWP, a domain containing a conserved proline-tryptophan-tryptophan-proline motif; PHD, a cysteine-rich region containing a plant homeodomain (with permission from Chen and Riggs 2005).

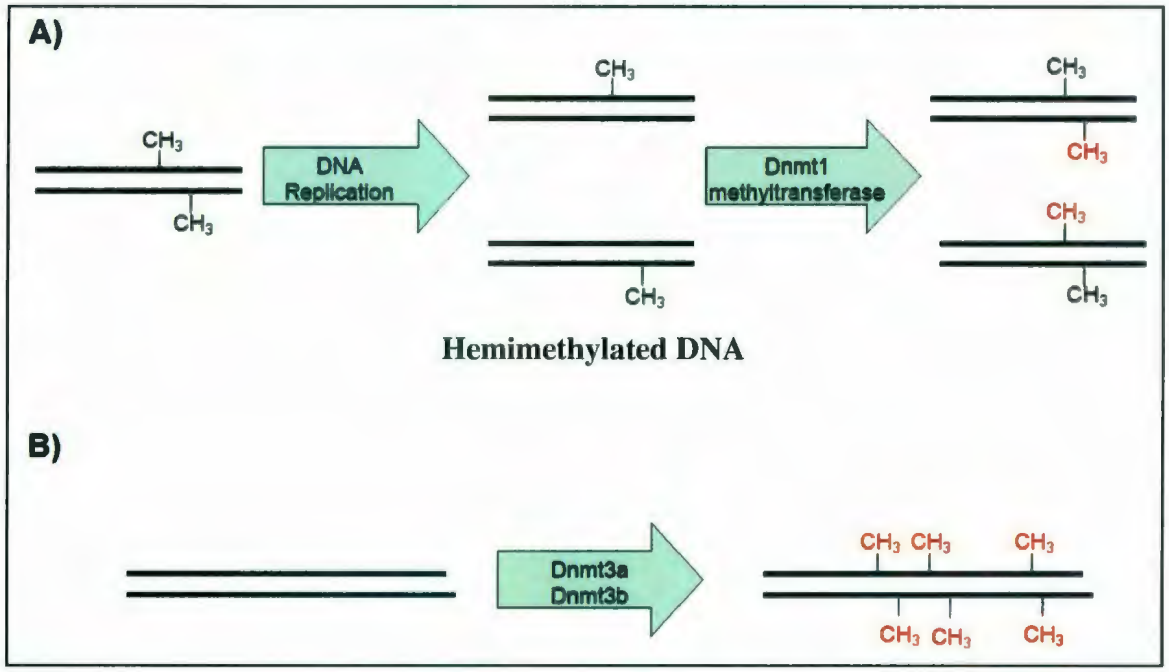


Figure 1.4 A) Dnmt1 preferentially methylates hemimethylated DNA and maintains the methylation pattern during DNA replication. B) Dnmt3a and Dnmt3b are capable of establishing methylation patterns in unmethylated DNA (Red methyl groups indicate newly added methylation events).

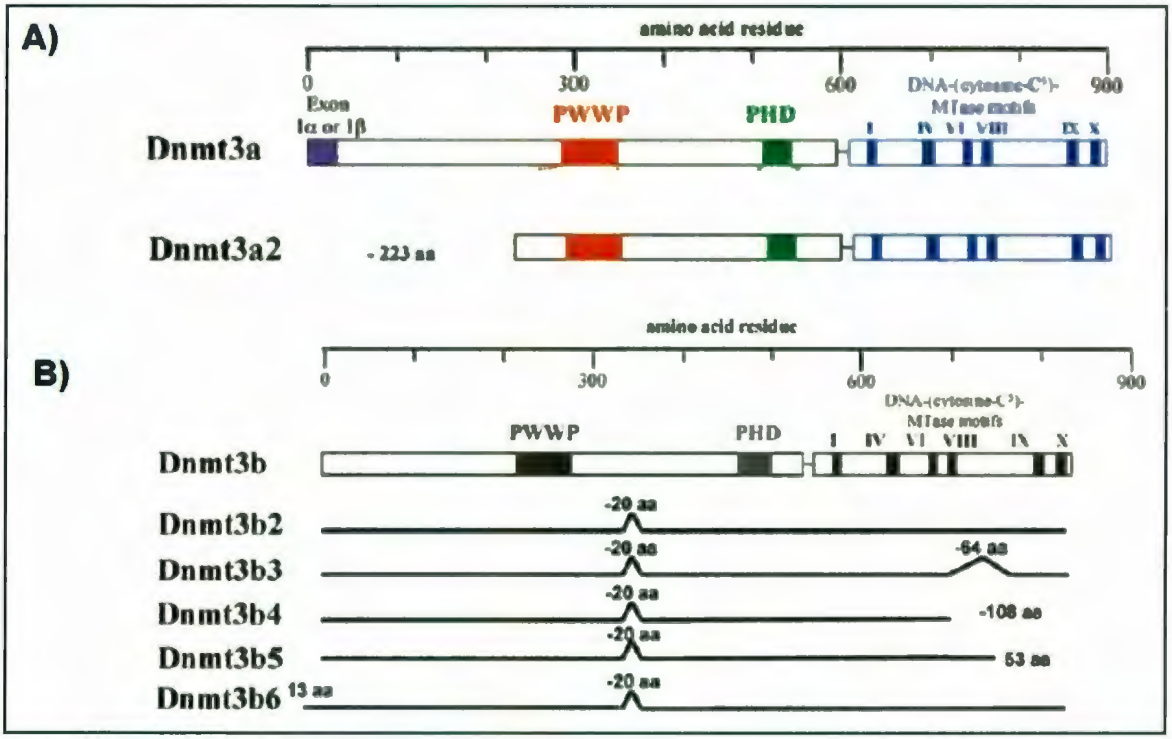


Figure 1.5 A) Schematic of mouse Dnmt3a and short isoform Dnmt3a2. Dnmt3a is more highly expressed in mammalian adult tissues while Dnmt3a2 is more highly expressed in embryonic development and targets euchromatin. B) Dnmt3b variants, several of which lack catalytic activity, are differentially expressed in development, and functional significance is unknown (adapted from Hermann *et al.* 2004).

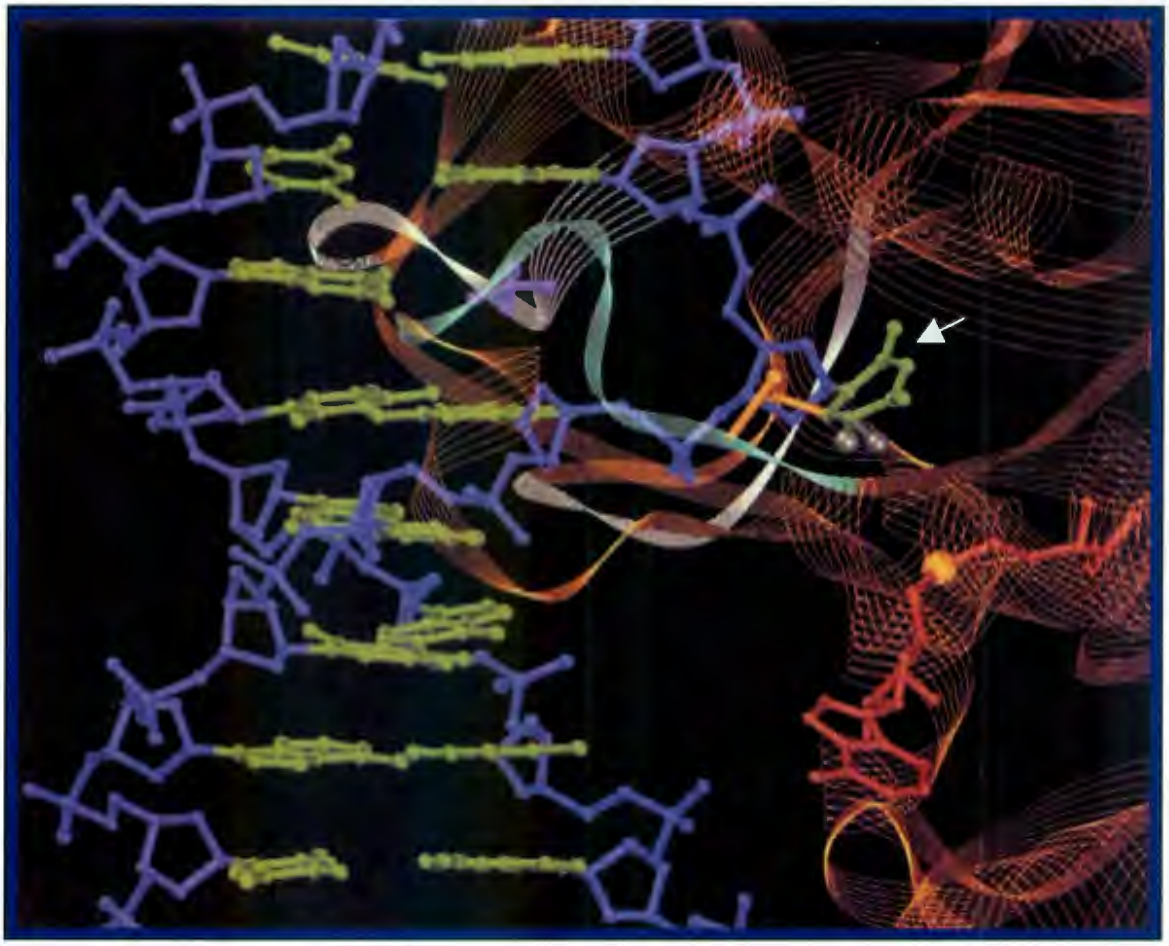


Figure 1.6 The mechanism of DNA methylation involves DNMT binding to DNA, everting the cytosine to project out of the double helix (arrow) and following nucleophile attack on cytosine carbon 6, a methyl group is transferred from the methyl donor SAM to carbon 5, followed by release (adapted from Voet and Voet 2004).

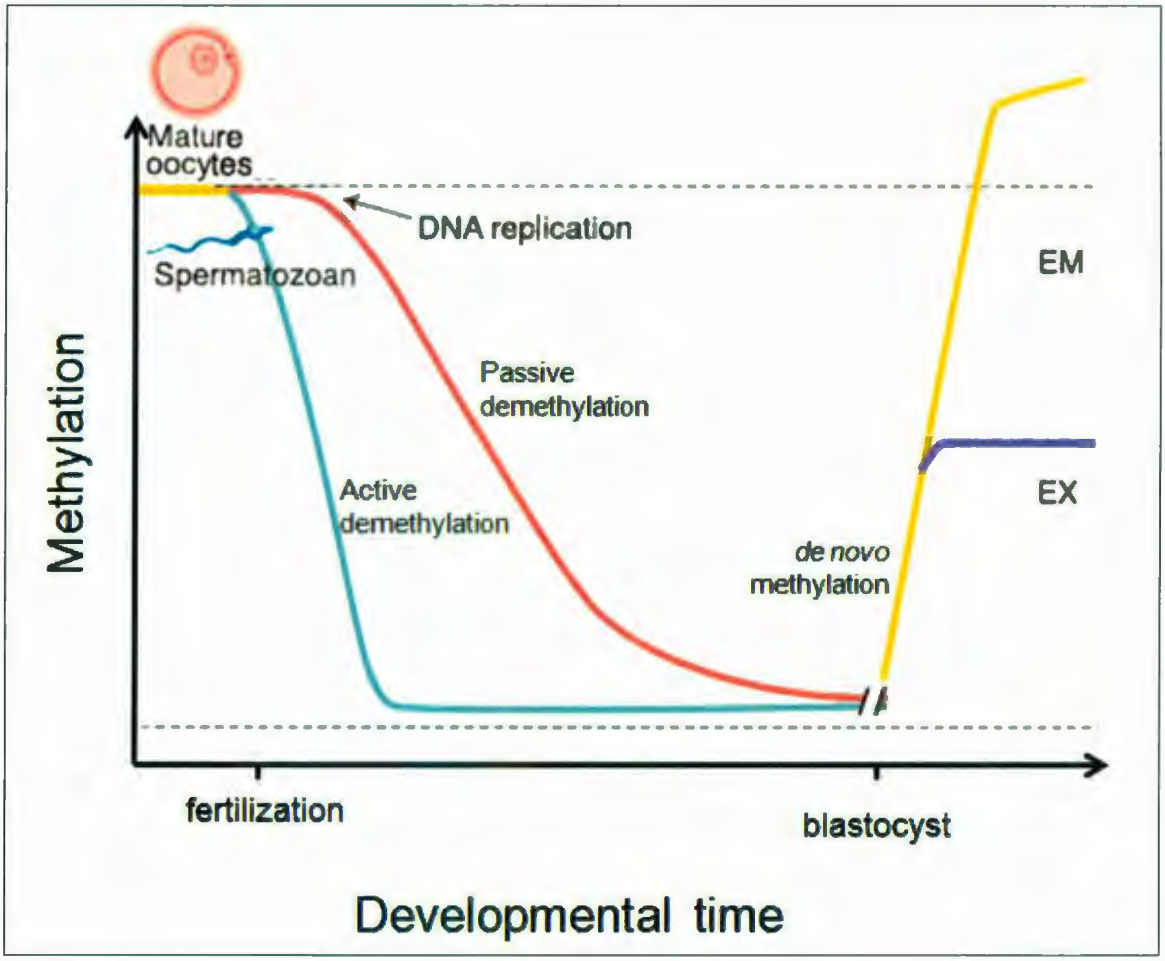


Figure 1.7 Methylation dynamics in mammalian development. The paternal genome (blue line) undergoes active demethylation following fertilization, while the maternal genome (red line) undergoes passive demethylation which is dependent on not maintaining methylation during DNA replication. At the blastocyst stage, a wave of *de novo* methylation occurs, though extraembryonic (EX) and embryonic (EM) lineages are methylated to different extents. Imprinted genes and some repeat sequences (dashed lines) maintain their methylation status throughout development (adapted from Reik *et al.* 2001).

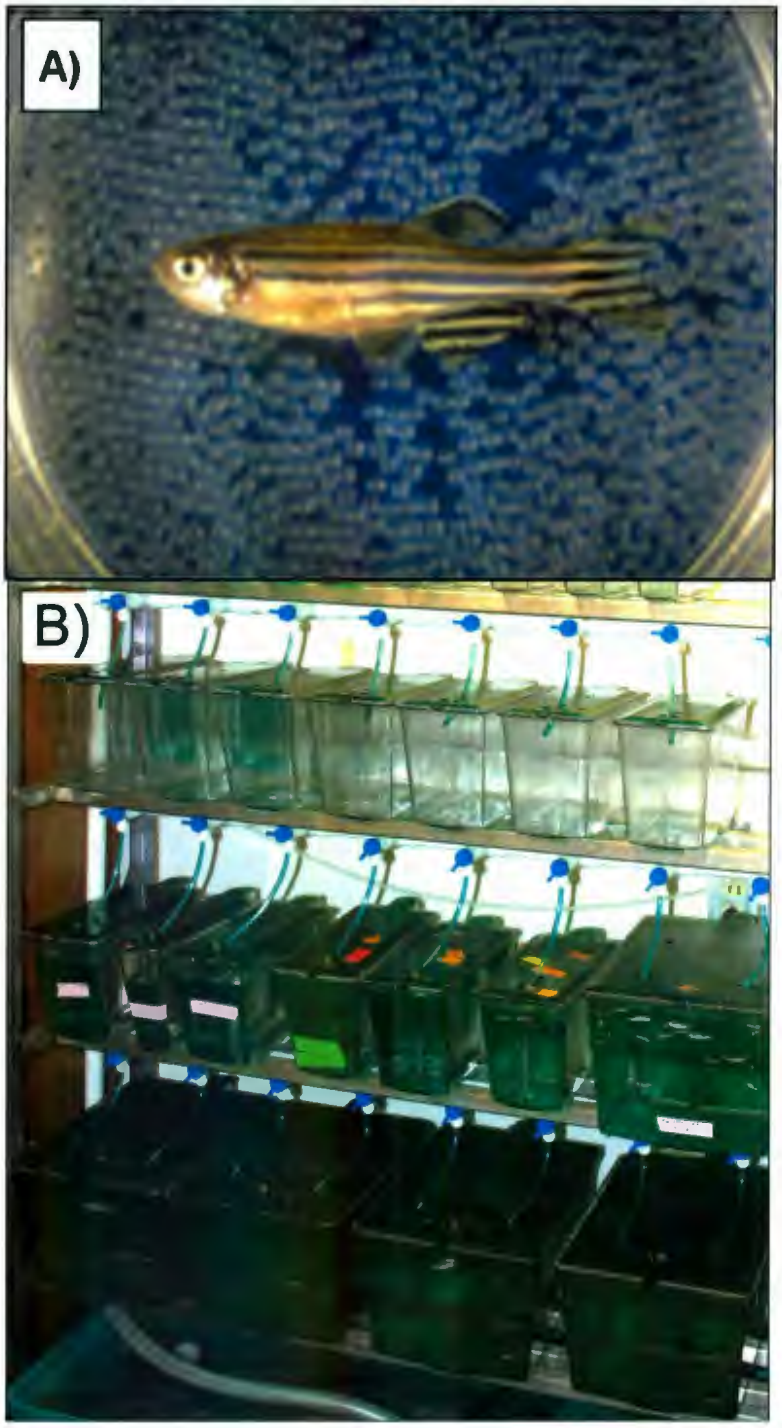
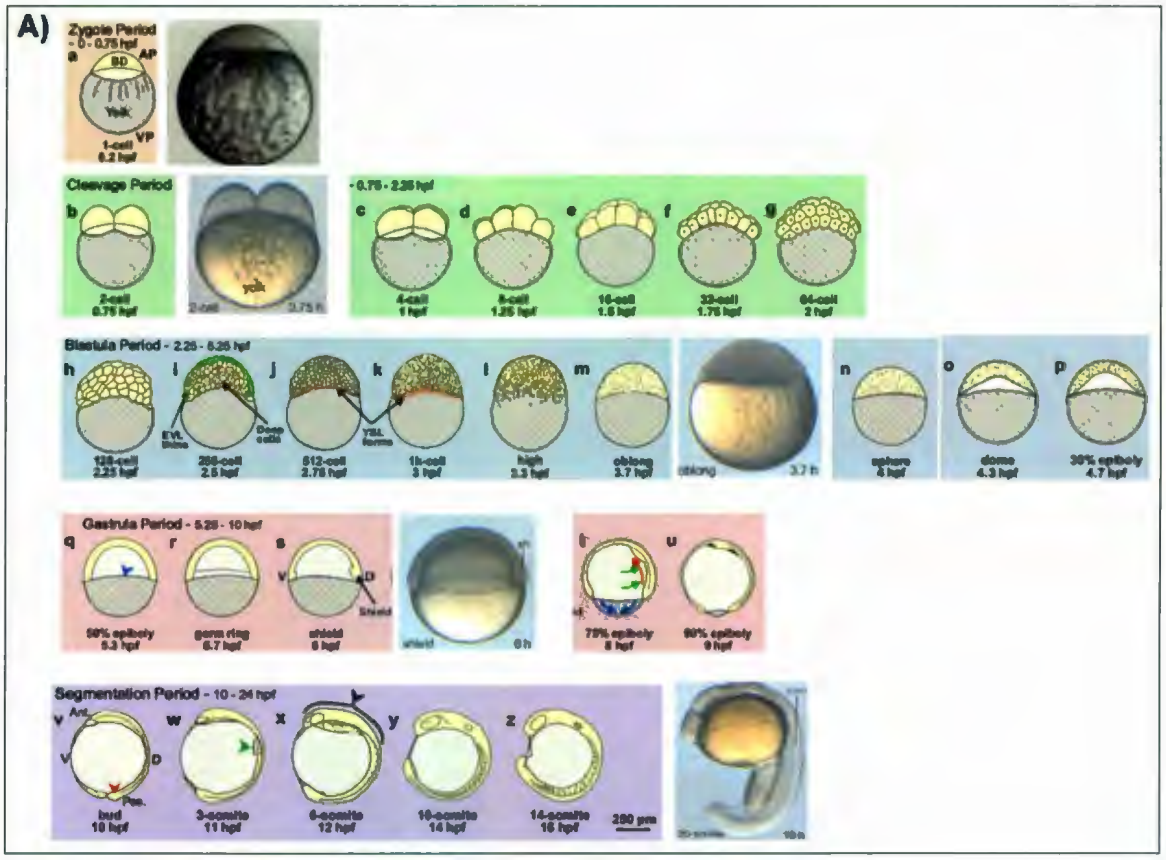


Figure 1.8 A) Female adult zebrafish in a 10 cm culture dish with hundreds of newly fertilized embryos (adapted from Megason and Link 2007). B) Housing for zebrafish in circulating system.


A)
Zygote Period
- 0 - 0.75 hpf
a
BD
AP
Yolk
VP
1-cell
0.2 hpf
Cleavage Period
b
2-cell
0.75 hpf
yolk
3.75 h
- 0.75 - 2.25 hpf
c
4-cell
1 hpf
d
8-cell
1.25 hpf
e
16-cell
1.5 hpf
f
32-cell
1.75 hpf
g
64-cell
2 hpf
Blastula Period - 2.25 - 5.25 hpf
h
128-cell
2.25 hpf
i
Evl.
Blstm
256-cell
2.5 hpf
j
Deep
cells
512-cell
2.75 hpf
k
YSL
forms
1k-cell
3 hpf
l
high
3.3 hpf
m
oblong
3.7 hpf
(oblong)
3.7 h
n
sphere
4 hpf
o
dome
4.3 hpf
p
30% epiboly
4.7 hpf
Gastrula Period - 5.25 - 10 hpf
q
50% epiboly
5.3 hpf
r
germ ring
5.7 hpf
s
V
D
shield
6 hpf
shield
6 h
t
75% epiboly
8 hpf
u
90% epiboly
9 hpf
Segmentation Period - 10 - 24 hpf
v
Ant.
V
D
Pos.
bud
10 hpf
w
3-somite
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z
14-somite
16 hpf
200 µm
10 h

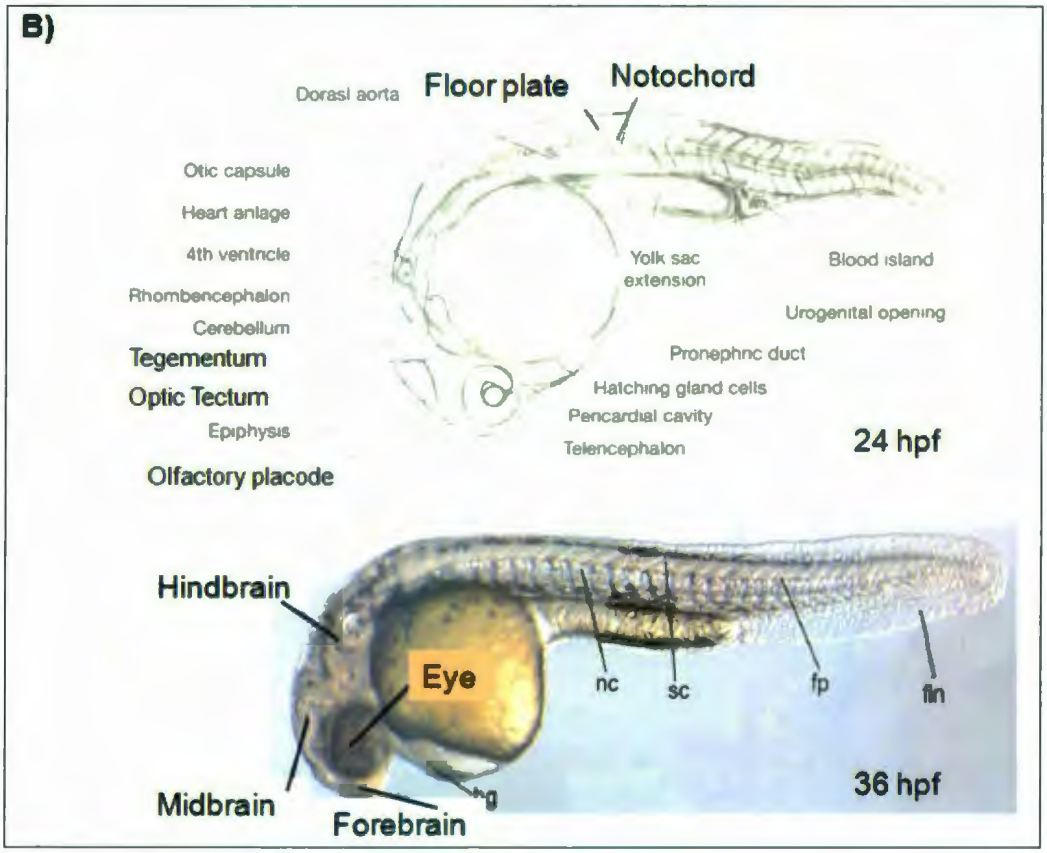

Figure 1.9 Zebrafish embryogenesis. A) Schematic and live embryo images of developmental stages from the zygote period to the mid-segmentation period (fertilization to 19 hpf). The dorso-ventral axis may first be distinguished at shield stage (6 hpf). Cell movements in gastrulation stage embryos of epiboly (blue arrows), convergence (green arrows), and extension (red arrows) are in panel t. D, V, AP, VP, EVL, Ant., Pos., and hpf are dorsal, ventral, animal pole, vegetal pole, enveloping layer, anterior, posterior and hours post fertilization, respectively (adapted from Webb and Miller 2007 and from Schier and Talbot 2005). B) Camera lucida drawing and live zebrafish image at 24 and 36 hours post fertilization, repectively. Structures of greater relevance to this thesis are in bold (adapted from Schier and Talbot 2005; Dahm 2002). hg, nc, sc, and fp, are hatching gland, notochord, sclerotome, and floor plate, respectively.

Chapter 2: Novel Splice Variants Associated with One of the Zebrafish *dnmt3* Genes

A version of this chapter has been published in BMC Developmental Biology (Smith, T.H.L, Dueck, C.C., Mhanni, A.A. and McGowan, R.A. (2005). Novel splice variants associated with one of the zebrafish *dnmt3* genes. BMC Developmental Biology 5(23)).

2.1 Introduction

The epigenetic modification of DNA by the addition of a methyl group to the 5 position of cytosine is an important mechanism for control of gene expression in vertebrates. This is particularly true during development where DNA methylation is thought to have a role in genome imprinting (Efstratiadis 1994; Reik and Allen 1994), X-chromosome inactivation (Riggs and Pfeifer 1992) and lineage determination (Cedar 1988). Methylation has been most intensely studied in mammals where the levels have been shown to be quite dynamic during early development, decreasing soon after fertilization and increasing again by the gastrula stage (Monk *et al* 1987; Santos *et al.* 2002). The importance of this de-methylation/re-methylation cycle to the developmental process has been clearly demonstrated by perturbations of that methylation that generally leads to embryonic lethality (Li *et al.* 1992; Okano *et al.* 1999). Given the importance of methylation in sustaining normal early developmental processes, the enzymes that add and maintain that methylation are of significant interest. The dnmt3 family of methyltransferases that are thought to be important in *de novo* methylation (that is the addition of methyl groups to previously unmethylated sequences) are of particular interest in this context. There are three members of this family in mammals; two have catalytic activity, Dnmt3a and Dnmt3b; and the third, Dnmt3l, is important as a cofactor, particularly for the methylation of imprinted loci (Hata *et al.* 2002). Functionally, however, the dnmt3 family is not limited to just three products because both the *Dmnt3a* and *Dnmt3b* transcripts can be alternatively spliced to generate a number of different RNAs. *Dnmt3a* has two splice variants differing in the 5' region whereas *Dnmt3b* has six

identified splice variants (Chen *et al.* 2003). These variations in the Dnmt3 transcripts and resulting proteins may allow for a greater diversity in the function and/or targets of these enzymes.

Methylation in zebrafish has recently been the focus of a number of reports, and methylation has been found to be dynamic during its early development (Mhanni *et al.* 2004). Also, as in mammals, blocking re-methylation in zebrafish results in abnormal development and death (Martin *et al.* 1999).

The zebrafish actually has at least twice the mammalian number of *dnmt3* genes; six have been submitted to databases so far (GenBank numbers AB196914, AB196915, AB196916, AB196917, AB196918, AB196919) (Figure 2.1) (Shimoda *et al.* 2005). The higher number of genes in this gene family in teleosts is not surprising due to the genome duplication event early in the teleosts lineage (Postlewait *et al.*, 1998). The significance of the increase in *dnmt3* gene copy number in zebrafish is unknown.

We have isolated and analysed a number of the zebrafish *dnmt3* gene sequences and have identified two *dnmt3* sequences that are located very close together in a single linkage group and are therefore likely to be the result of a tandem gene duplication event. The very close proximity of the two sequences provides an interesting opportunity to examine how the expression of these genes is controlled since one copy has a very limited upstream promoter region relative to the other.

2.2 Methods

Total RNA was isolated from ovarian tissue using the phenol/chloroform method of Chomczynski and Sacchi (Chomczynski *et al.* 1987). Ovarian Poly A+ RNA (FastTrack 2.0 kit, Invitrogen Inc. Carlsbad, CA) was used for first strand cDNA synthesis using the BD SMART™ RACE cDNA Amplification Kit (Clontech, Palo Alto, CA) according to the Manufacturer's instructions. Using the zebrafish dnmt3 EST (GenBank number AF135438), a gene specific primer, GSP1 (see Table 1 for primer sequences), was designed to amplify, along with the universal primer, the relevant cDNA using PCR conditions as described by the manufacturer. Based on a resulting sequence that corresponded to four different regions of the genomic map (The Sanger Institute Welcome Trust zebrafish sequencing project), a series of gene specific primers were designed for four different paralogues.

PCR products were fractionated on 0.8% agarose gels, visualized with ethidium bromide, excised from the gel and cloned into pCR 2.1 vectors (TOPO TA cloning kit, Invitrogen Inc. Carlsbad, CA). The cloned products were then purified (Wizard®Plus Minipreps, Promega Inc. Madison, WI), and sequenced (Cortec DNA Service Laboratories Inc., Kingston, ON).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) was used to determine the relative expression levels of gene 1 (*dnmt3*), gene 2 (*dnmt5*), and its variants in tissues. Total RNA from zebrafish ovarian tissue, 1–2 cell embryos, 64 cell embryos, 6 hour embryos, muscle tissue, and brain tissue was isolated as described above, and the integrity checked by ethidium bromide staining. The RNA was then reverse

transcribed using M-MLV Reverse Transcriptase (Invitrogen Inc. Carlsbad, CA) according to the manufacturer's instructions and using primers specific for the various genes and variants. GSP2 was used for first strand cDNA synthesis of Gene 1 in conjunction with GSP3, generating a predicted amplicon of 521 bp. Primer GSP4 designed to anneal to all three gene 2 variants was used with GSP5 for dnmt3-2-1 to produce a 420 bp amplicon and with GSP6 to produce two amplicons of 597 bp and 675 bp from gene 2 variants dnmt3-2-2, and dnmt3-2b, respectively. In addition, RT-PCR was conducted to generate a 440 bp amplicon with GSP7 and GSP8, primers specific for a constitutively expressed transcript, *max* (Schreiber-Agus *et al.* 1994). PCR reactions were set up as described by the manufacturer, except that 2 ul of cDNA template were used for each reaction. PCR conditions were designed to ensure that all amplifications were within the logarithmic phase. Those conditions were: 94°C for 1 min, 25 cycles of (94°C 30 sec, 59°C for 30 sec, 72°C for 1 min), and a 72°C for 1 min final extension for all primer sets except *max* which was only amplified for 14 cycles. Controls lacking RT were run for each RNA sample.

RT-PCR products were separated on a 1.5 % agarose gel, transferred to nylon membrane (Roche, Indianapolis, IN) and visualized by hybridization with a biotin labeled sequence designed to hybridize to gene 1, gene 2, and the variants (North2South Biotin labeling kit, Pierce Biotechnology Inc. Rockford, Il). Densitometric analysis of autoradiographs was performed to determine the relative expression levels of the transcripts and their splice variants at the above mentioned zebrafish developmental stages and tissues. Samples could be compared on different blots by using a control

sample present on each autoradiograph, and samples were calibrated using the endogenous control *max*.

Zebrafish care and feeding were performed essentially as described by Westerfield (Westerfield 1995). All experimentation was done with the approval of the Canadian Council on Animal Care.

2.3 Results and Discussion

We used a *dnmt3* sequence already present in the zebrafish EST database (GenBank number AF135438) to identify and isolate the complete cDNA sequences of four of the *dnmt3* genes found in the zebrafish. Three of these are located in the same linkage group (linkage group 23) and two of them very closely juxtaposed to each other (Figure 2.2). The very close proximity of those two genes has some interesting implications with respect to their origin and the control of their expression, given the much more limited potential promoter region of one relative to the other. We, therefore, undertook a closer examination of the two genes, which we named *dnmt3-1* and *dnmt3-2*.

From the end of the polyA addition site of dnmt3-1 to the beginning of our cloned sequence for dnmt3-2 (probably not actually beginning at the cellular transcriptional start site) consists of only 1428 base pairs. Since there is only a small amount of 5' sequence that is associated with the dnmt3-2 gene this limits the control of the expression of this gene to a small and easily manipulated region. Analysis of this region suggests that it is a TATA-less promoter with a number of potential transcription factor binding sites including AP1 and SP1 binding sites, which have also been reported for mammalian

*Dnmt3*s (Yanagisawa *et al.* 2002; Detich and Szyf 2005). The sequence of the cloned genes, *dnmt3-1* and *dnmt3-2,* revealed open reading frames that could encode polypeptides of 1447 and 1297 amino acids, respectively. Comparison of the sequences of these two genes to zebrafish genomic maps present in the Genbank database allowed for an analysis of the genomic structure. That structure, along with the relative position of the two genes, is shown in Figure 2.2. The two genes are very similar in sequence; 72% identical at the nucleotide level and 74% identical at the amino acid level, with large regions being more than 80% identical (Figure 2.3). This is in contrast to only 19–28% identity at the nucleotide level, and 36–46% amino acid similarity when compared to the other *dnmt3* sequences present in the zebrafish genome. This trend is also true for the conserved methyltransferase motifs. For instance, the PWWP motif of dnmt3-1 and dnmt3-2 are 88% and 84% similar at the amino acid and nucleotide levels, respectively, but considerably less similar to the other *dnmt3* sequences (e.g. *dnmt3-2* vs. gene 4 (dnmt7), accession #196918, has 64% and no significant similarity at the amino acid and nucleotide levels, respectively) (BLAST 2 Sequences, NCBI).

Recent additions to the sequence databases included two zebrafish sequences that appear to correspond to the same two genes and were named *dnmt3* and *dnmt5* respectively (GenBank numbers AB196914, and AB196916, respectively). Our sequencing data corroborate the sequences submitted to the databases except for a few minor variations in regions with triplet repeats which may be an artefact of polymerase slippage in cloning or represent real triplet repeat differences that exist in the gene.

The high similarity between *dnmt3-1* (*dnmt3*) and *dnmt3-2* (*dnmt5*) relative to other zebrafish *dnmt3*s, as well as their close proximity, suggests that these genes arose from a

duplication event. Postlethwait *et al.* (1998) provides support for a model where two polyploidization events occurred in a common ancestor of zebrafish and mammals. However, there are often additional multigene members in zebrafish. Postlethwait *et al.* (1998) argues that either chromosome duplication or another tetraploidization event in the zebrafish lineage is the most likely mechanism by which these additional members arose. The tight clustering seen here, however, suggests that, at least in this instance, tandem gene duplication has occurred.

The most interesting aspect of our analyses is that at least one of the genes, *dnmt3-2* (*dnmt5*), includes at least two start sites and a number of splice variants. These were initially identified in cDNA libraries generated from 1–2 cell embryos and RACE-PCR and were later confirmed by RT-PCR in a number of early embryonic zebrafish stages as well as somatic tissues (Figure 2.4). This demonstrates that they are all expressed at least to the level of RNA. Densitometric analysis revealed that the transcript levels are not equivalent and that the relative levels of the different genes and isoforms fluctuate independently between the stages examined (Figure 2.5). All genes and variants examined are expressed in early embryonic stages, though *dnmt-3-2-1* appears to be the most significant prior to zygotic gene activation (zygotic gene activation occurs at ~3 hours post fertilization). All transcripts demonstrated declining levels leading up to this event, suggesting maternal supply turnover. Following zygotic gene activation, however, there appears to be a marked shift towards *dnmt3-1* being the most highly expressed. Additionally, there appears to be tissue dependent differences in transcript expression levels (Figure 2.5 B). These differences in expression profiles for the different transcripts

and splice variants suggest that they are regulated independently and each may be playing distinct and separate roles during the development of the zebrafish.

The shortest of these variants, *dnmt3-2-1*, corresponds to the *dnmt5* sequence in the database. The two novel variants reported here differ in size from that sequence by 187 (*dnmt3-2-2*) and 265 (*dnmt3-2-2b*) base pairs. These variants are actually associated with the gene having the most restricted promoter region. A schematic of the three products is shown in Figure 2.6.

There are several interesting aspects of these *dnmt3-2* (*dnmt5*) variants. To begin with, although the splicing difference between variant *dnmt3-2-2* and *dnmt3-2-2b* appears to involve the same 3' splice junction, it has a different 5' splice junction, meaning that one of those splice sites is located within the exon of the other variant. However, both of the junctions still abide by the GT/AG rule for splice junctions.

The second interesting aspect of these splice variants is that all of them are 5' to the initiator AUG. Therefore, none of them actually affect the amino acid sequence. This suggests that either the splicing differences are trivial or they play a regulatory role in the translation or localization or some other aspect of the expression of various splice variants. The latter possibility is a more reasonable assumption since, parsimoniously, it seems unlikely to assume that this RNA would be alternatively spliced in a variety of ways for no biologically relevant reason. This situation is not unique to zebrafish *dnmt3* genes. Similar splice variants in the 5' untranslated region have also been reported for human *DNMT3*s, though the significance of these variants is not known (Yanagisawa *et al.* 2002).

2.4 Conclusion

We have isolated and analysed several of the *dnmt3* genes from the zebrafish. In this report we have focused on two of the genes that are located in close proximity in a single linkage group and we find that the two genes are considerably more similar to each other than they are to the other zebrafish *dnmt3* genes. This suggests that they arose as a result of a relatively recent gene duplication event. We have also found evidence for the existence of several different splice variants and alternative splice sites associated with one of the two genes, reminiscent of what is seen with the human *DNMT3*s. Expression analyses of these genes and variants demonstrate they are dynamic during development with distinct patterns that suggest they are independently controlled and, possibly, have different functions in development.

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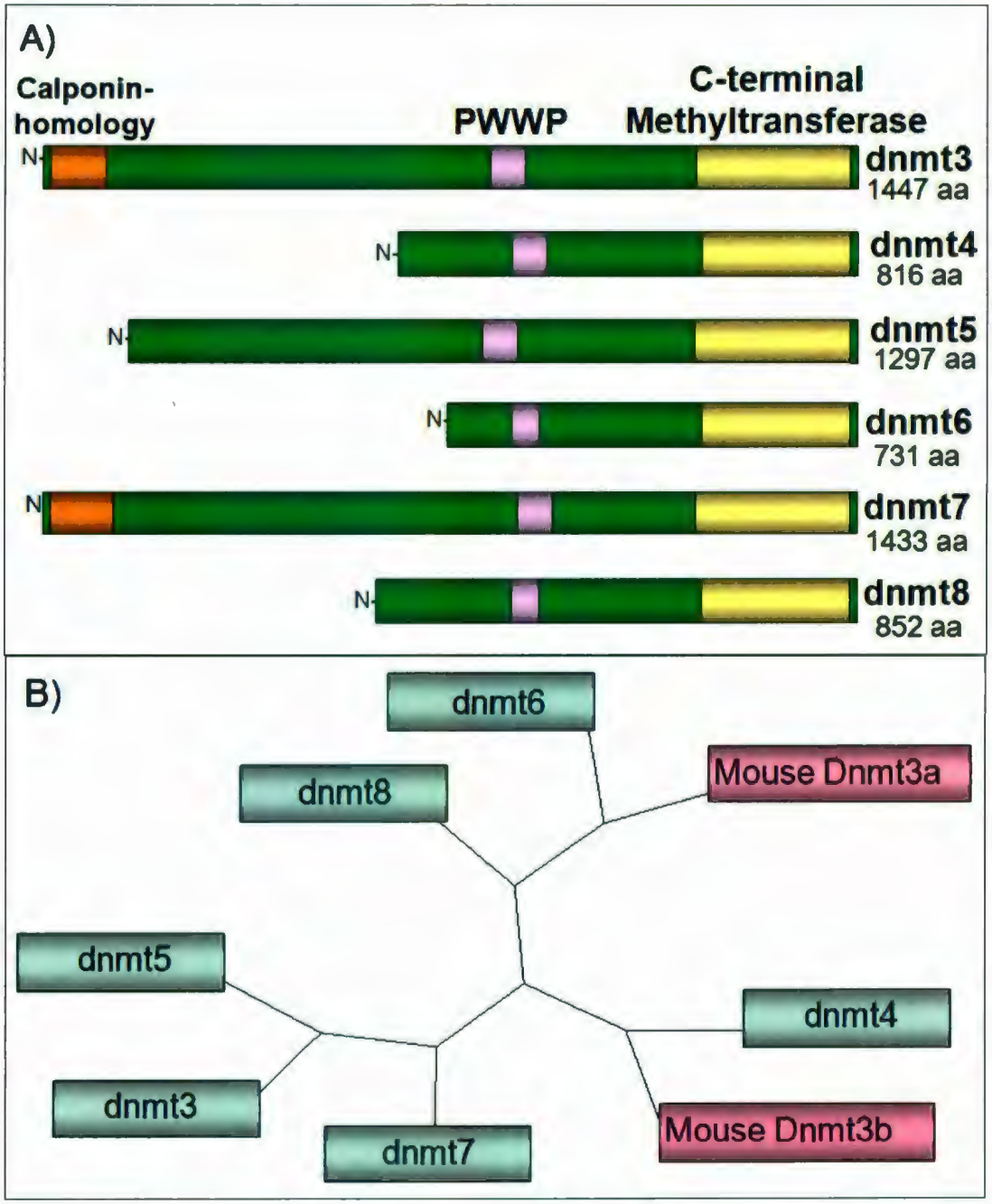

Figure 2.1 The *de novo* methyltransferase family in zebrafish. A) Schematic of zebrafish dnmt3 proteins. B) Radial Tree showing relationships between amino acid sequence of zebrafish DNA methyltransferases and mouse Dnmt3a and Dnmt3b (also in Shimoda *et al.* 2005).

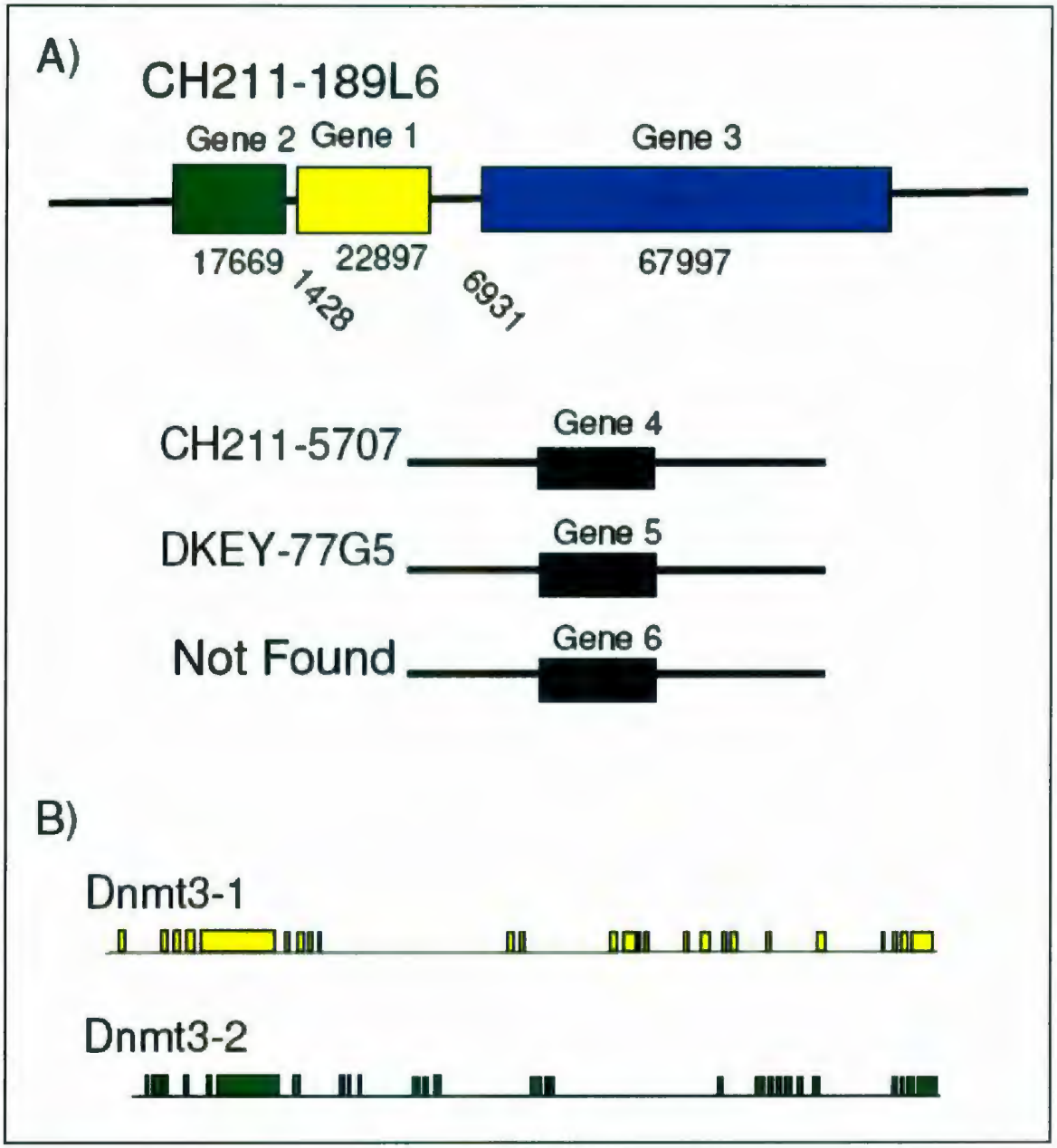

Figure 2.2 The *dnmt3* genes in zebrafish. A) The Welcome Trust Sanger Institute library numbers are provided for all genes while the genomic size and distances are indicated for genes 1, 2, and 3. B) A more detailed view of the genomic structures of Gene 1 (*dnmt3*) and Gene 2 (*dnmt5*). Boxes represent exons and adjoining lines represent introns.

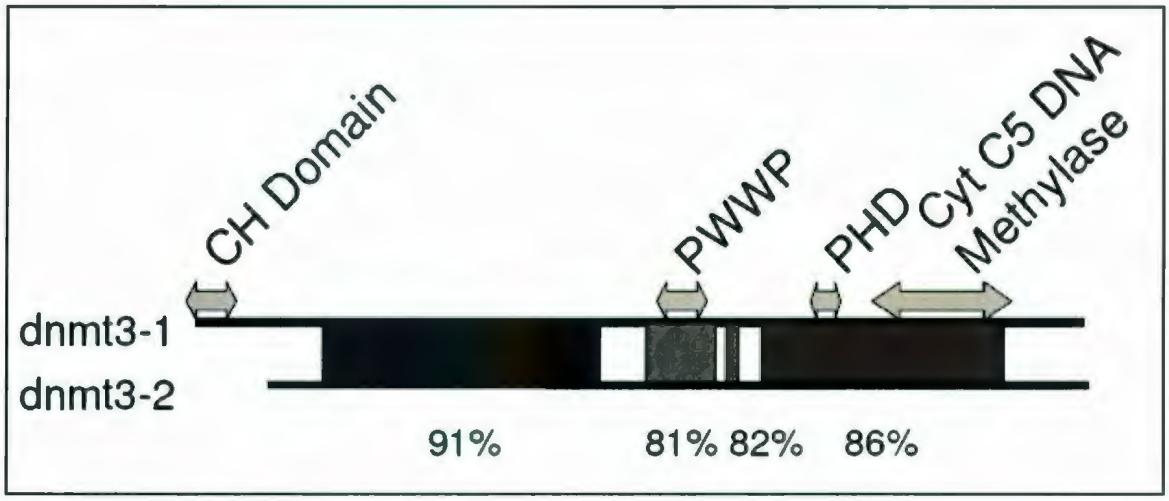

Figure 2.3 Homology between Gene 1 (*dnmt3)* and Gene 2 (*dnmt5)* at the nucleotide level. Percentages above 80% are shaded, and specific homology percentages are provided below the figure (numbers obtained by BLAST alignment, NCBI). Arrows span the nucleotides that give rise to the identified motifs labelled above the respective arrows. Note: All identified motifs are characteristic of the C-5 methyltransferases, with the exception of the CH domain.

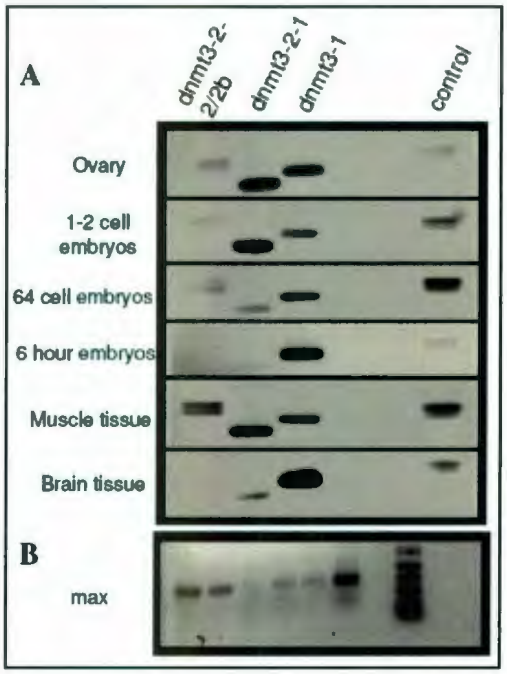

Figure 2.4 *dnmt3* isoforms. A) RT-PCR followed by agarose gel electrophoresis and hybridization with a biotin-labelled probe. Stages/tissues used are labelled on the left side of figure. The first lane in all cases contains a doublet representing the two splice variants of dnmt3-2 differing in size by 78 bp. The second lane shows the alternate translational start site variant of dnmt3-2. The third lane is the product of the primers specific for gene 1 and the last lane is a control reaction loaded on each gel to allow comparisons between gels. The amount of reaction loaded was not the same in all lanes but was varied to produce more equivalent band intensities for more accurate quantification. Controls lacking reverse transcriptions produced no amplification products (not shown). B) RT-PCR of a constitutively expressed gene, *max*, was performed for each RNA used serving as an internal standard for quantification. Lanes 1–6 show the *max* amplicons generated from the samples used in panel (A), ovaries through to brain. Lane 7 contains size markers.

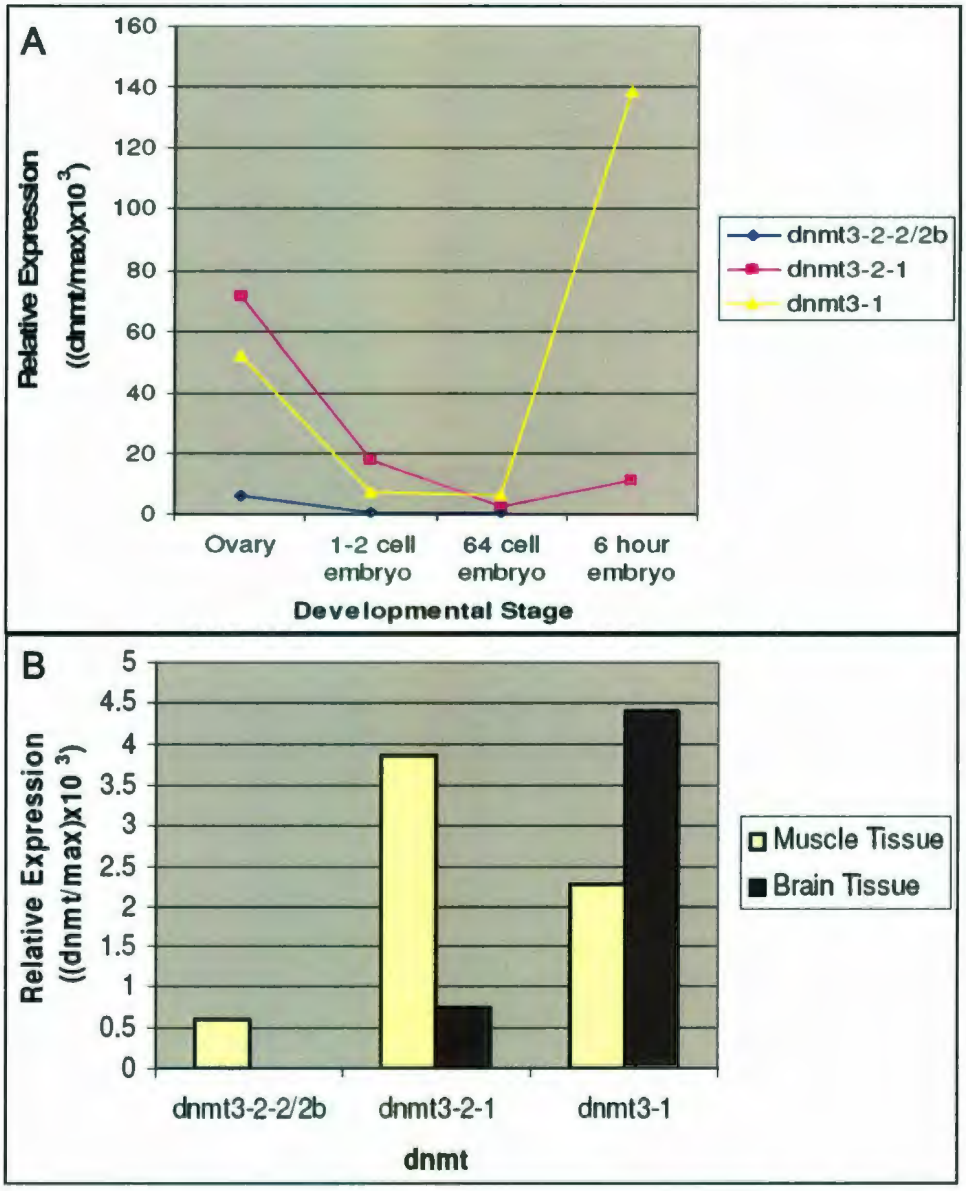

Figure 2.5 Expression summary. A) Graph showing data from Figure 2.4 developmental stages corrected for differences in amounts loaded, and normalized to *max* to correct for concentration differences as well as the control for exposure differences (see methods). B) Graph showing data from Figure 2.4 somatic tissues corrected as above and demonstrating the relative expression levels of the three transcripts in those tissues.

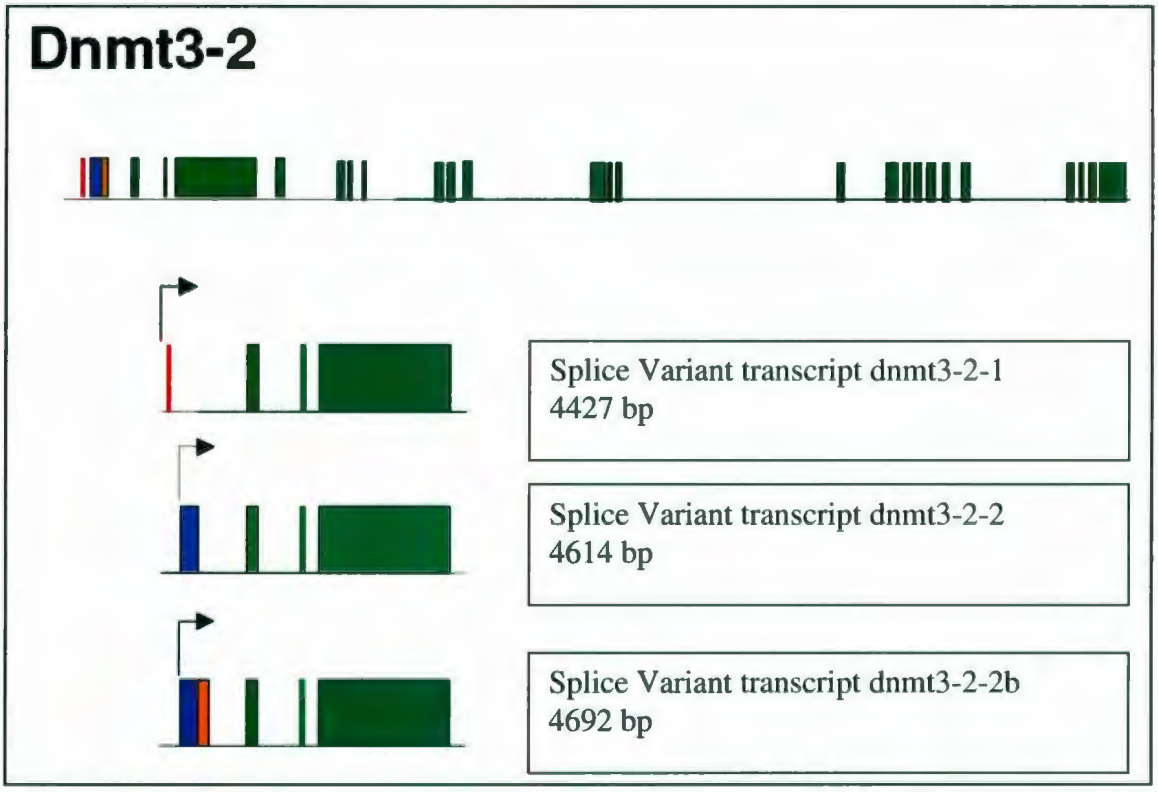

Figure 2.6 The various transcripts produced from Gene 2 (*dnmt5*). The genomic structure is presented on the top line, with the 5' region of interest magnified below illustrating the various alternative splicing products. Splice variant dnmt3-2-1 differs from the others by an alternate transcriptional start site and a missing exon 2. Transcripts dnmt3-2-2 and dnmt3-2-2b lack the first exon and are alternatively spliced in the second exon (the one missing from transcript 1). All splice variants occur upstream of the AUG start site (Red box = exon1, blue and orange box = exon2).

Table 2.1 Primers used.

Primer name	Sequence
GSP1	5'- GACAGGACCCTGAATGGACGTCGCT
GSP2	5'- GAGAGAGCACTGAGATGTCAG
GSP3	5'- CCAGAAATCTGTTGGAGACATTACACC
GSP4	5'-AAGGCAGTATGGAGTCTGTCTGCA
GSP5	5'-CAGTCATGGCAATGTCTTTCC
GSP6	5'-ATGTATGTCCTGTGAGGAGGAAC

Chapter 3: Expression of *de novo* DNA Methyltransferases in Zebrafish Development

3.1 Introduction

In eukaryotes, the addition of a methyl group to the 5 position of cytosine found in CpG dinucleotides is catalyzed by the DNA methyltransferase (Dnmt) proteins (Brero *et al.* 2006). In mammals, the Dnmts are comprised of Dnmt1, Dnmt2, Dnmt3a, Dnmt3b, and Dnmt3l. However, the activities of the enzymes are divided into distinct families, where Dnmt1 is important for the maintenance of methylation patterns during DNA replication, while the Dnmt3 family of proteins are referred to as the *de novo* methyltransferases due to their ability to establish the methylation pattern on DNA during development (Brero *et al.* 2006). Dnmt2, unlike the others, may not be relevant to DNA methylation but rather to RNA methylation instead (Rai *et al.* 2007).

DNA methylation is a repressive mark that has been implicated in cell differentiation (Li *et al.* 2007), modulating tissue-specific gene expression (Oka *et al.* 2005; Rai *et al.* 2006), genomic imprinting (Kaneda *et al.* 2004), X-chromosome inactivation (Barr *et al.* 2007), silencing of endogenous parasitic elements (Walsh *et al.* 1998), cancer (Esteller *et al.* 2001), learning and memory, and mental health disorders (Mill *et al.* 2008). DNA methylation is not universal to all animals, though it appears to be essential for proper development in those that do employ this mechanism of gene control (Suzuki and Bird 2008). For instance, and for reasons still under investigation, *Dnmt3b* and *Dnmt3a* null mice demonstrate either pre- or early post-natal lethality, respectively (Okano *et al.* 1999; Suzuki and Bird 2008). Although the relevance of DNA methylation to the development of zebrafish, a non-mammalian vertebrate, has been debated in the past, mounting evidence indicates that in fact, as in other animals which

methylate, it is very relevant and likely even essential (Martin *et al.* 1999; Smith *et al.* 2005; Rai *et al.* 2006; Mackay *et al.* 2007).

There are several questions concerning methylation that remain unresolved. These include, but are not limited to, understanding the precise role(s) of each of the Dnmts in development as well as in the above mentioned processes, and uncovering what it is that enables the Dnmts to target particular DNA sequences, given the apparent lack of sequence preference beyond CpG dinucleotides (Chen and Li 2006).

Study of DNA methylation during development highlights a particularly intriguing phenomenon, as methylation levels are highly dynamic during the early stages (Morgan *et al.* 2005). Traditionally, mammals, and in particular, mice, have been the favoured models for methylation studies. However, in these animals the embryos are least accessible when many of these changes are occurring. Zebrafish on the other hand, have been well established as an excellent model system for developmental studies given their external fertilization, optically clear embryos, and rapid ontogeny (Link and Megason 2008).

In addition to being an excellent developmental model for organogenesis, zebrafish demonstrate significant potential for investigations of DNA methylation. We and others have shown that several aspects of zebrafish methylation are in keeping with mammals. For instance, the dynamic changes in methylation that are seen during the course of mammalian embryonic development have also been evidenced in zebrafish embryos (Mhanni and McGowan 2004; Mackay *et al.* 2007). Additionally, the pattern of methylation in zebrafish is described, as in mammals, as global methylation (Suzuki and Bird 2008). Here, DNA methylation is located throughout the genome and punctuated

with short unmethylated regions termed CpG islands. This is in contrast to many plants, fungi, and some invertebrates, where methylation patterns have a fractional organization and are said to be mosaic, with heavily methylated and unmethylated domains. The functional utilization of DNA methylation also appears to differ between these systems (Brero *et al.* 2006; Suzuki and Bird 2008).

Another attribute of zebrafish that may help in gaining an understanding of the role of methylation during development is that zebrafish, unlike mammals, do not have imprinted genes (Yokomine *et al.* 2006). Zebrafish do not have an identified Dnmt3l, which acts as a co-factor to Dnmt3a in methylating mammalian imprinted genes. This is not a surprise as the attainment of a Dnmt3l gene in a common ancestor of mammals through gene duplication has been suggested to potentially be a critical event in the evolution of imprinting (Yokomine *et al.* 2006). However the lack of the confounding factors of dnmt3l and imprinting in zebrafish may facilitate teasing out the function(s) of the various dnmts in development. This is particularly true for Dnmt3a because it will allow us to identify its role in development, beyond its role in imprinting.

All of the *dnmt* genes have been isolated from the zebrafish and one difference between them and mammals is in the number of *dnmt3* genes. Specifically, six different *dnmt3* genes have been identified in zebrafish, along with splice variants for *dnmt5* (Shimoda *et al.* 2005; Smith *et al.* 2005). It is not unusual, however, to observe multiple paralogues in zebrafish relative to mammals. There are numerous examples in zebrafish where this is the case, and the observed redundancy is a result of a genome duplication event during the evolution of teleosts estimated to have occurred approximately 450 million years ago (Postlewait *et al.*,1998). Sequence analysis of the six zebrafish *de novo*

methyltransferase genes suggests that four of the six (*dnmt3*, *dnmt4*, *dnmt5*, *dnmt7*) are more closely related to DNMT3B (amino acid identity 50%-67%), while two of the zebrafish *dnmts* are more similar to DNMT3A (*dnmt6* and *dnmt8*) (amino acid identity 81% and 76%, respectively). Interestingly, and in keeping here, Dnmt3a has been more highly conserved in vertebrate evolution, while Dnmt3b is more divergent. Likely, Dnmt3a serves a fundamental role in vertebrate development, and in placental animals was co-opted for imprinting (Yokomine *et al.* 2006). We have previously suggested that *dnmt3* and *dnmt5* represent a relatively recent tandem gene duplication event (Smith *et al.* 2005).

Here we examine the transcript expression of the zebrafish *de novo* methyltransferases during development, and in adult muscle and brain tissues. Both semi-quantitative RT-PCR with templates from eight developmental stages and *in situ* hybridization analysis of two developmental stages have been carried out in order to examine the transcriptional control of the *dnmt3* genes. By characterizing the transcript expression of these genes we are able to begin identifying the stages in development and the different tissues where the various *dnmts* may be more functionally relevant.

3.2 Methods

Reverse transcription-polymerase chain reaction (RT-PCR) was used to determine the relative transcript expression levels of zebrafish genes *dnmt3* (Genbank accession # AB196914), *dnmt4* (Genbank accession # AB196915), *dnmt6* (Genbank accession # AB196917), *dnmt7* (Genbank accession # AB196918) and *dnmt8* (Genbank accession #

AB196919). Total RNA was isolated from adult zebrafish ovarian, muscle, and brain tissues, and seven developmental stages spanning 1-2 cell to 72 hour stage embryos. Total RNA was isolated using the phenol/chloroform method of Chomczynski and Sacchi (Chomczynski *et al.* 1987). The RNA was reverse transcribed with M-MLV Reverse Transcriptase (Invitrogen Inc. Carlsbad, CA) according to the maufacturer's instructions and with 1 ug RNA. PCR was carried out using primers specific for the various genes (Table 1) and that spanned more than one exon to determine genomic contamination. In addition, RT-PCR was conducted to generate a 440bp amplicon with primers specific for a constitutively expressed gene, *max* (Schreiber-Agus *et al.* 1994). PCR conditions were designed to ensure that all amplifications were within the logarithmic phase. Those conditions were; 94°C for 1 min, 26-28 cycles of 94°C 30 sec (cycle number was gene dependent and *max* was amplified for 16 cycles), 59°C for 30 sec, 72°C for 1 min, and a 72°C for 1 min final extension for all primer sets. Each developmental stage sample consisted of a minimum of 400 embryos, and three independent samples were collected and analysed for expression per developmental stage. Muscle, brain, and ovary samples were dissected from adult fish and were performed in triplicate. The same proportion of of PCR product was run on agarose gels for each stage or tissue examined (From 50 ul PCR product, 24 ul target gene PCR product, and 12 ul *max* PCR product was loaded for agarose gel electrophoresis). No RT controls were carried out alongside each reaction.

RT-PCR products were separated by electrophoresis on a 0.8% agarose gel and visualized by SYBR Green staining (Roche Mississauga, ON). Densitometric analysis (Scion image, NIH) of gels using mean pixel intensities was performed to determine the relative expression levels of the transcripts for the zebrafish developmental stages and

tissues examined. Samples were normalized using the endogenous control *max* by determining the ratio of *max*/target gene ratio of the densitometric analysis (Schreiber-Agus *et al.* 1994).

Embryo fixation and DIG-labelled *in situ* hybridization were performed as described by Thisse and Thisse (2007). The antisense and sense probes for the zebrafish *dnmt*s correspond to the following nucleotides: *dnmt3* & *dnmt5*, 864 nt (AB196914 nucleotides 1028-1891; probe 100% match to *dnmt3,* 98% match to *dnmt5*); *dnmt7*, 896 nt (AB196918 nucleotides 5039-5934), *dnmt6* & *dnmt8*, 715 nt (AB196917 nucleotides 1713-2427; probe 100% match to *dnmt6,* 79% match to *dnmt8*). Probes were also designed to more specifically target *dnmt3* alone and *dnmt6* alone, though results were similar to those obtained from the *dnmt3* & *dnmt5*, and *dnmt6* & *dnmt8* probes.

Zebrafish care and feeding were performed essentially as described by Westerfield (Westerfield 2000). All experimentation was done with the approval of the Canadian Council on Animal Care.

3.3 Results and Discussion

RT-PCR revealed that zebrafish *dnmt* transcript levels are dynamic during development and that they demonstrate tissue specificity (Figure 3.1 and Figure 3.2). The presence of transcripts in early cleavage stages in all cases presumably represents a maternal supply of *dnmt* RNA and the general decrease in transcript levels that is seen prior to zygotic gene activation is suggestive of maternal supply turnover (Figure 3.2). This is followed by an up-regulation shortly after the mid-blastula transition (MBT) stage

which occurs just prior to three hours post-fertilization during zebrafish development when raised at 28.5 °C. Interestingly, general trends of transcript expression following the MBT divide the genes into two groups, and these groupings also are in keeping with genes most similar to mammalian *Dnmt3b* and those most similar to mammalian *Dnmt3a*. *dnmt3*, *dnmt4*, *dnmt5* (not shown here, but previously reported; Smith *et al.* 2005), and *dnmt7* demonstrate a more pronounced increase in transcript expression around 6 hpf of development, decline by 24 hpf, and are no longer detectable, at this PCR cycle number, by 72 hpf (Figure 3.2). This is in contrast to *dnmt6* and *dnmt8*, which demonstrate early expression with up-regulation following the MBT continuing through to at least 72 hpf (Figure 3.2).

In addition to the difference in regulation seen following 6 hpf between the zebrafish genes homologous to *Dnmt3b*, and those homologous to *Dnmt3a*, the former genes were not as highly expressed in adult zebrafish muscle and brain as levels shown during embryogenesis. Conversely, *dnmt6* and *dnmt8* demonstrate equal or higher levels of transcript expression in the adult brain relative to their developmental regulation (Figure 3.2).

In situ analysis at 24 hours provides information on the tissue specific regulation of these genes during development. One region of shared expression among the *de novo dnmt*s during development at 24 hours is in the optic tectum (Figure 3.3). This structure in non-mammalian vertebrates is the major visual processing center of the brain, and interestingly, zebrafish are capable of retinotectal regeneration (Bilotta and Saszik 2001).

Transcripts corresponding to the *de novo dnmt* genes are certainly expressed in the head region and brain more so than caudally during zebrafish development and, with

exception of *dnmt6*, *dnmt8*, and *dnmt4, de novo dnmt* expression appears to be limited to this region. At 24 hours, *dnmt3* and *dnmt5* are expressed in the telencephalon, diencephalon, hindbrain, eye, and brachial arches (Figure 3.3). *dnmt7* on the other hand appears to be most strongly expressed in the optic tectum and tegmentum (Figure 3.3). *dnmt6* and *dnmt8* demonstrate a more ubiquitous expression throughout the embryo (Figure 3.3). Expression of *dnmt6* and *dnmt8* appears less intense in the notochord, however, at 24 hpf this tissue becomes progressively less penetrable to RNA probes for whole-mount *in situ* hybridization (Thisse and Thisse 2007) and may not be indicative of a decrease in expression. *dnmt4* at 24 hours shows a punctuated expression along the aorta wall as well as its expression in the eye and brain (midbrain, rhombomere 4) (published elsewhere Thisse and Thisse 2004).

Although it is important to realize zebrafish whole-mount *in situ* analyses beyond 48 hpf are no longer indicative of expression in the trunk and tail due to lack of RNA probe penetration (Thisse and Thisse 2007), *in situ* results at 72 hours corroborate RT-PCR findings with negligible expression of *dnmt3*, *5*, and *dnmt7* (Figure 3.4) in tissues previously demonstrating expression and that are penetrable at this stage. *dnmt4* also shows declining expression, and by the long-pectoral stage expression is limited to the retina proliferative zone, posterior tectum, brain ventricular zone, and branchial arches (Thisse and Thisse 2004). *dnmt4* expression beyond 48 hours of development was not determined. *In situ* background levels at 72 hours (multiple probes tested) against *dnmt6* and *dnmt8* prevent conclusions regarding tissue specific expression at this time point (Figure 3.4), though RT-PCR findings suggest relatively higher levels of expression than at 24 hours for *dnmt6*, and at least equal levels of expression for *dnmt8* (Figure 3.2).

Overall, the differences in *in situ* expression of the *de novo dnmt*s in one to three day embryos demonstrates that these genes are regulated independently and may, therefore, be playing distinct roles during development of the zebrafish.

De novo dnmt expression in zebrafish shows some strong similarities to the expression of the *Dnmt3*s in mammals. Generally, in mammals, *Dnmt3a* and *Dnmt3b* are expressed in ES cells, early embryos and developing germs cells, and are downregulated in somatic tissues of postnatal animals (Okano *et al.* 1999). In the central nervous system, however, recent evidence indicates an initial overlapping expression of *Dnmt3b* and *Dnmt3a* during early neurogenesis (E11.5-E15.5), after which *Dnmt3b* becomes undetectable (Feng *et al.* 2005). *Dnmt3a* on the other hand, becomes significantly increased in expression levels postnatally, reaching peak levels during the first three postnatal weeks, and decreasing again by adulthood. This peak in expression corresponds to timing of neural maturation in mice. The authors conclude that both *Dnmt3a* and *Dnmt3b* are likely important during early neurogenesis, but *Dnmt3a* probably has a role in CNS maturation and postnatal function (Feng *et al.* 2005). Interestingly, in zebrafish, neuronal maturation is occurring between 24-72 hpf (Detrich *et al.* 1999), a time period where we demonstrate *dnmt6* and *dnmt8* expression is relatively high.

3.4 Conclusion

This study demonstrates that the *de novo* methyltransferases in zebrafish display dynamic expression patterns during development, and exhibit some similarities to general expression patterns in mammalian development. All transcripts are likely maternally

supplied. The genes most similar to *Dnmt3b* are relatively less expressed following somitogenesis, while genes most similar to *Dnmt3a* are relatively highly expressed through organogeneis and neurogenesis stages. Additionally, these latter genes are relatively prevalent in adult tissues examined. Overlapping, as well as tissue specific expression was demonstrated by *in situ* analysis, suggesting the *de novo dnmt*s may have some redundancy, or interaction dependent roles in development, as well as non-overlapping specificities.

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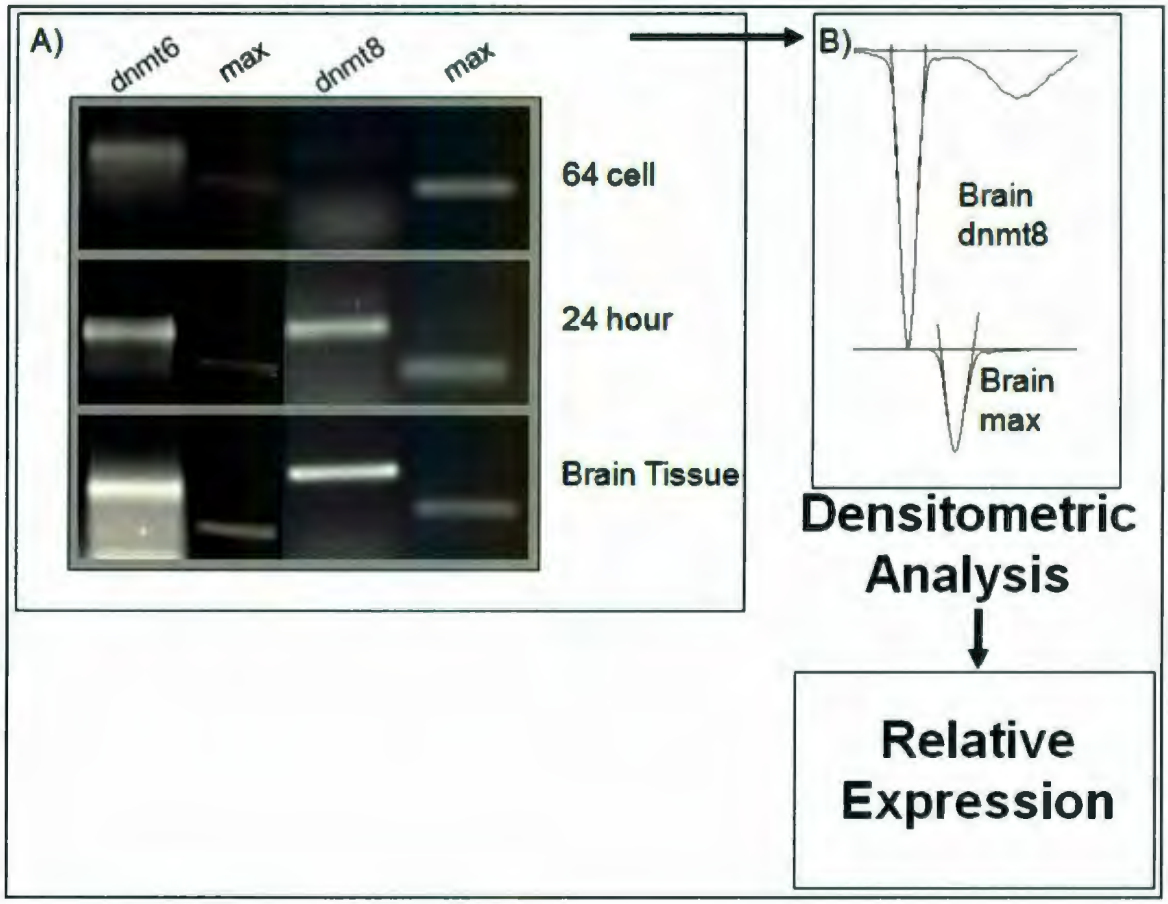

Figure 3.1 *dnmt3* gene expression determined by A) RT-PCR, followed by gel electrophoresis, SYBR staining, and B) densitometric analysis. Shown here, sample data of *dnmt6* and *dnmt8* alongside ubiquitously expressed control gene *max* in 64 cell stage embryos, 24 hour embryos, and adult brain tissue.

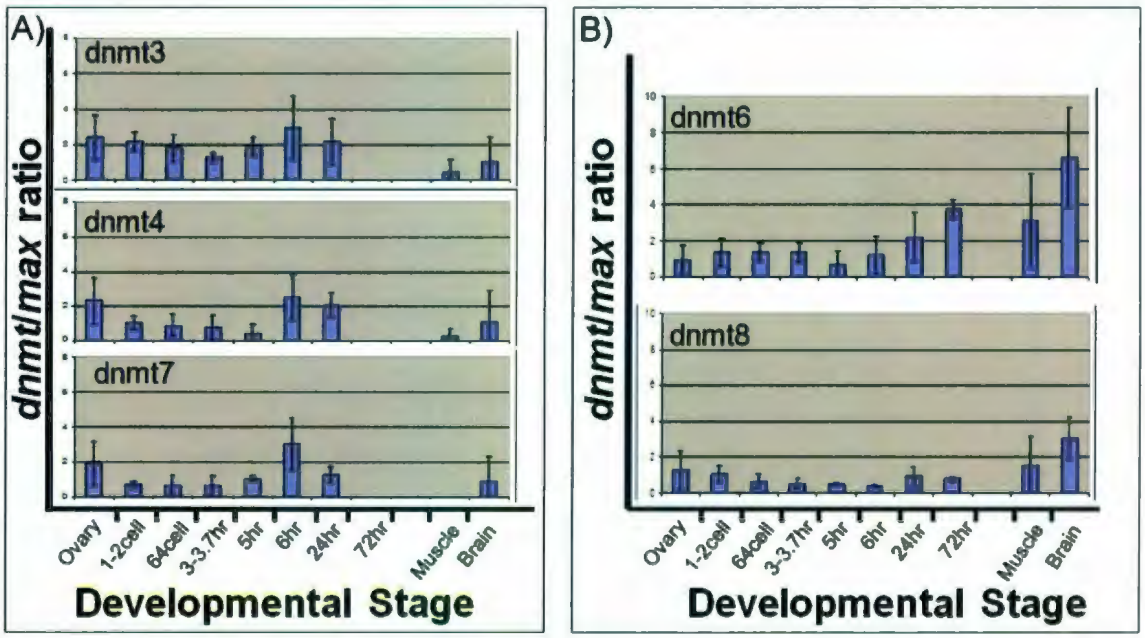

Figure 3.2 Relative expression levels of *dnmt3* genes during zebrafish embryonic development and in adult tissues. Expression data represent the average expression ±SD determined from three biological replicates. In the case of developmental stages, samples consist of a minimum of 400 embryos collected per sample. General trends of expression divide the genes into two groups. A) *dnmt3B*-like genes that demonstrate increased expression at 6 hours development, and no longer detectable at this PCR cycle number by 72 hours and relatively low levels in muscle and brain B) *dnmt3A*-like genes that present equal or increasing expression through to 72 hours of development, and relatively high levels of expression in adult muscle and brain.

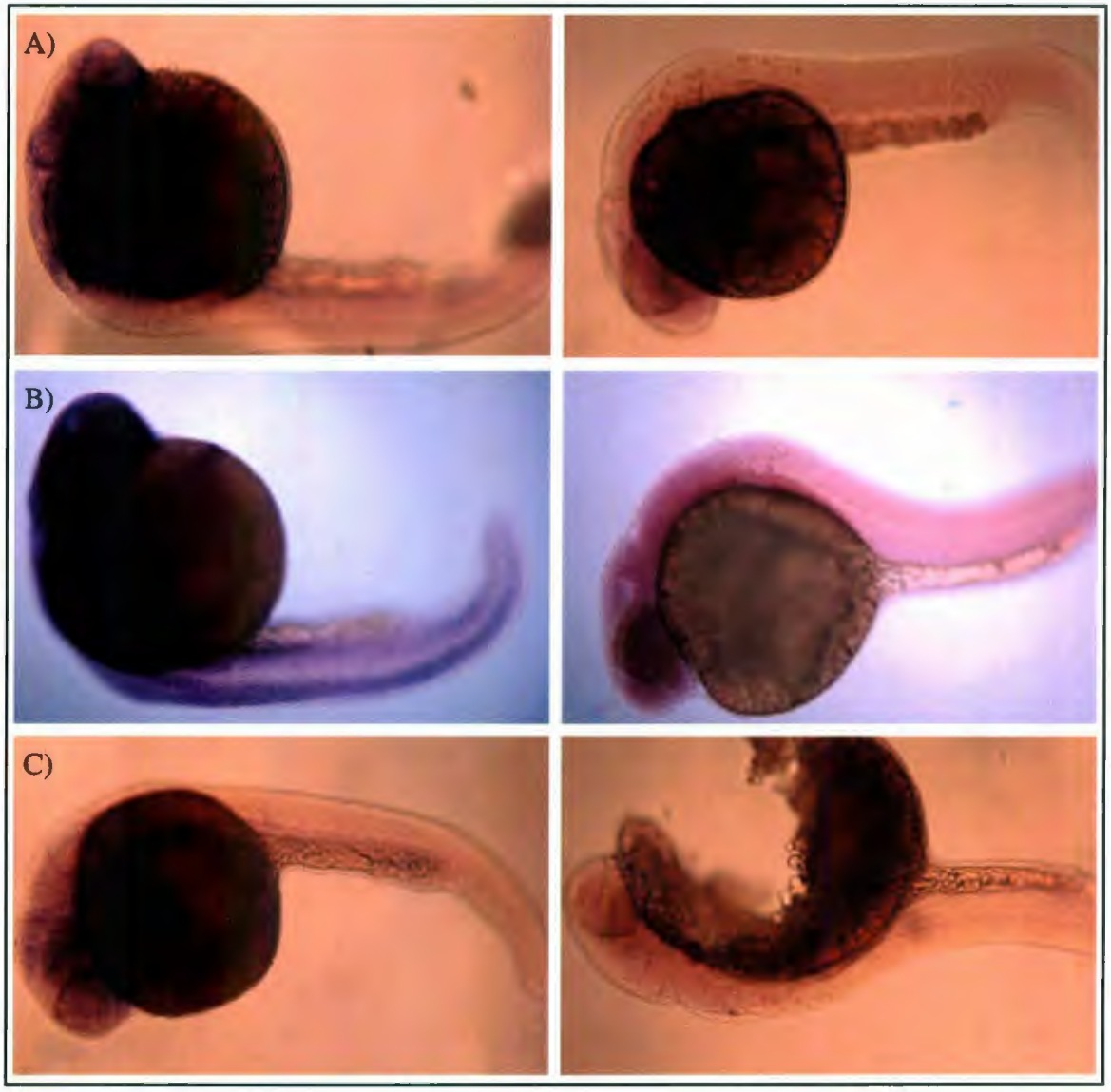

Figure 3.3 Analysis of *dnmt* expression by whole mount *in situ* hybridization at 24 hours development. Panels on the left are hybridized with antisense riboprobes, and panels on the right are hybridized with sense control riboprobes. A) probe designed against *dnmt3 & dnmt5* demonstrates expression in the fore-, mid-, and hindbrain. B) *dnmt6 and dnmt8* demonstrate expression more ubiquitously, though some background is present in sense control embryos. C) *dnmt7* is expressed in the optic tectum and tegmentum.

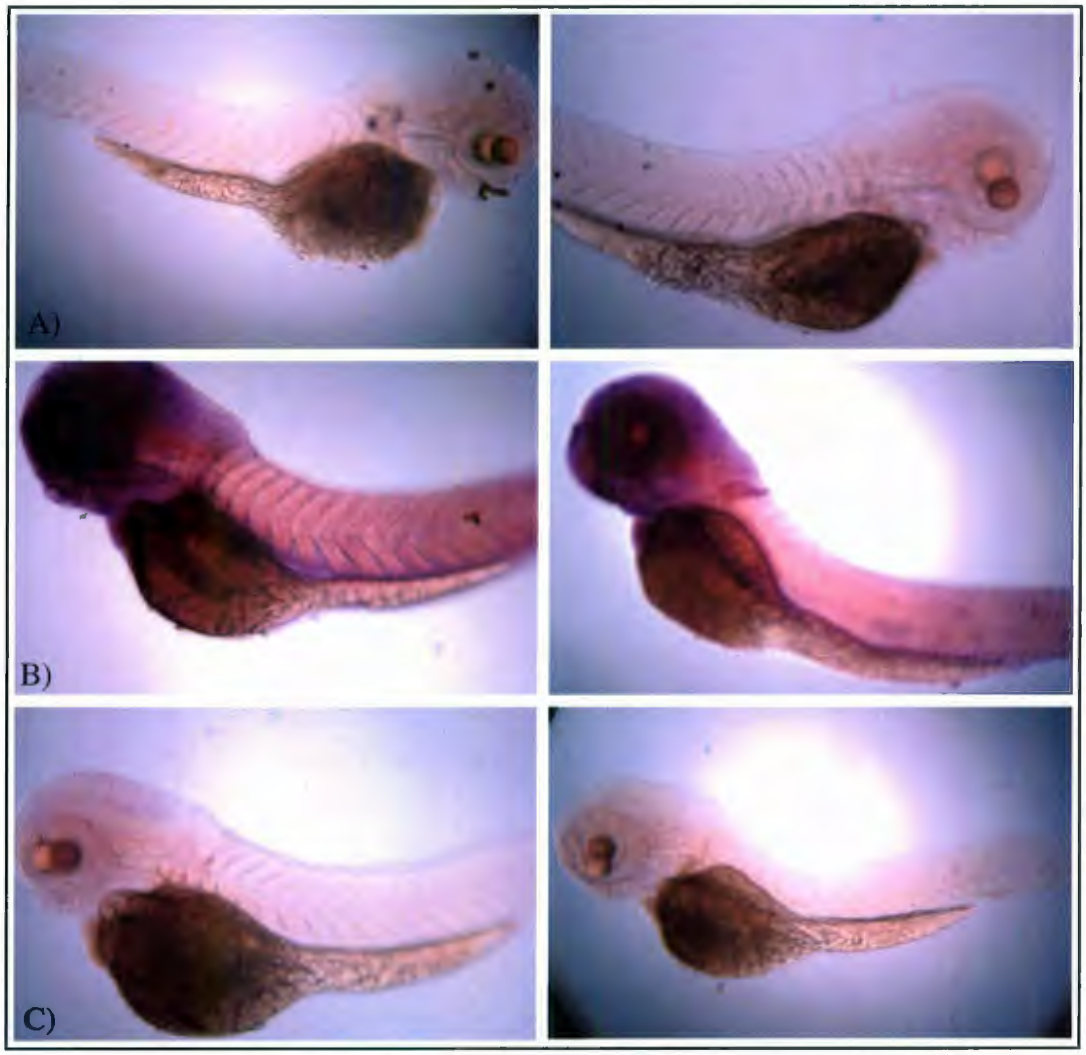

Figure 3.4 Analysis of *dnmt* expression by whole mount *in situ* hybridization at 72 hours development. Tail and trunk tissues are impenetrable to RNA probes at this stage. Panels on the left are hybridized with antisense riboprobes, and panels on the right are hybridized with sense control riboprobes. A) probe designed against *dnmt3 & dnmt5* indicates expression levels undetectable. B) *dnmt6 and dnmt8* background levels in sense control embryos at 72 hours make determining expression by *in situ* difficult despite attempting the use of several different probes. C) low expression of *dnmt7* is detected in the optic tectum.

Table 3.1 RT-PCR primers employed to generate gene specific amplicons.

Primer Name	Sequence (5'- 3')	Amplicon size
dnmt3F	ATGGACATCATTCAACCTGG	543 bp
dnmt3R	AAGGCAGTATGGAGTCTGTCTGCA	
dnmt4F	CTCCACTACTGAAACAGCCAGTC	587 bp
dnmt4R	GTTCTCTGCTGTAGACCTCCTCA	
dnmt6F	AGAGCAGGCAAAACCTTCC	553 bp
dnmt6R	CCAACAAATCCACACACTCC	
dnmt7F	TGCAATCCTGTGATGATTGATGC	382 bp
dnmt7R	AGAGATGTCGGATCACAGGAACG	
dnmt8F	CACGTATGTTGGAGACATCAGG	595 bp
dnmt8R	GTTGGACACGTCAGTGTAATGG	
maxF	GCCGAAGAATGAGCGACAACG	440 bp
maxR	GCTTGTCCCTTGGGGTTGGTG	

Chapter 4: Knockdown of Zebrafish *de novo* DNA Methyltransferases

4.1 Introduction

Many inroads to understanding DNA methyltransferases and their function in development are being made through the use of morpholino antisense oligonucleotide (MO) injections into zebrafish embryos. This reverse genetic approach allows for the study of particular genes of interest during early development. MOs are small (~25 base pairs) synthetic DNA analogues containing a 6-membered morpholine ring instead of a deoxyribose moiety; they have a neutral charge backbone, and are resistant to nuclease cleavage (Chen and Ekker 2004). The conserved spacing and similar base-stacking ability of MOs as compared to natural genetic material allows the MO to undergo complementary base pairing to the transcript of interest and inhibit its function (Figure 4.1) (Nasevisius and Ekker 2000).

Based on design, two different types of MO are commercially available (GeneTools, LLC), and disrupt gene function in one of two ways (Figure 4.2). The first type of MO acts as a translation blocker and may be targeted to sequence located between the 5'cap and the first 25 bases 3'of the translation initiation codon (Chen and Ekker 2004). The presence of the start site blocking MO sterically hinders the binding of the translation machinery, resulting in knockdown of the protein of interest. Given the mRNA level of the target gene is unaffected, efficacy of knockdown may be assessed by western blotting provided appropriate antibodies are available (Chen and Ekker 2004).

The second type of MOs, splice-site targeting MOs, target exon-intron junctions, sterically hinder the splicing apparatus, and may effectively cause either an exon to be skipped and excluded or an intron to be included (Figures 4.2 and 4.3) (Ekker and Larson

2001). The concentration of MO injected may need to be higher for this type of MO relative to translation blocking MOs due to the narrow window during which nuclear processing events take place, and therefore the brief time period where they may be effective (Malicki *et al.* 2002). As MO interference results in aberrantly spliced transcripts, the efficacy of the knockdown may be assessed by designing primers flanking the splicing event. The presence of an aberrant transcript may be determined by RT-PCR, clearly an important advantage as it removes the need for appropriate antibodies (Figure 4.3) (Chen and Ekker 2004). However, maternally provided transcripts will remain unaffected as they are already processed.

One of the major advantages of zebrafish over other models of development is that zebrafish embryos are particularly amenable to reverse genetic approaches (Link and Megason 2008). Large numbers of embryos may be collected (typical pairing may provide 50-250 eggs) and up to a thousand embryos may be injected in a matter of hours. Injections are performed using a pressure injector, micromanipulator, and a dissecting microscope (Figure 4.4). Preferably, injections are performed on 1-cell stage embryos to ensure even distribution of the morpholino; however, zebrafish embryos are meroblastic during early development, allowing for reagent transfer between cells until the 16-cell stage (Link and Megason 2008). Embryos are then allowed to develop normally, and may be monitored for phenotypes resulting from the knockdown (Figure 4.4). As morpholinos may not result in complete loss of expression, the term "knockdown" is used rather than "knockout," and embryos with a phenotype are referred to as "morphants" rather than "mutants" (Link and Megason 2008). Of course, monitoring for morphants is made possible by the transparent nature and rapid ontogeny of zebrafish embryos and

further molecular analysis of the knockdown and its effects may be easily pursued as embryos with or without phenotype may be collected, *in situ* hybridizations may be performed, protein may be extracted for western blots *etc.*, or nucleic acids may be extracted for a number of analyses, such as Northern blots, RT-PCR, and bisulfite sequencing.

A precaution that needs to be considered when designing and performing zebrafish MO experiments is that, despite relatively low toxicity compared to other reverse genetic technologies, non-specific off-target effects do occur with 15-20% of MOs used in zebrafish (Urtishak *et al.* 2003; Robu *et al.* 2007). Non-specific effects include neural death that peaks at 1 day post-fertilization, and embryos having smaller heads and eyes and exhibiting somite and notochord abnormalities, as well as demonstrating craniofacial defects later in development (Robu *et al.* 2007). The cause of these effects is p53-induced apoptosis; interestingly, they may be alleviated with injection of p53-targeted MOs. One approach that is proving quite effective is to co-inject a p53 MO with MOs that result in off-target effects, allowing resolution of specific loss of function phenotypes (Robu *et al.* 2007).

In addition to the above mentioned "morpholino" phenotype that needs to be monitored, Ekker and Larson (2001) highlight four scenarios that need to be considered when injecting morpholinos, underlining the critical necessity for proper controls. The first scenario is desired affect which is the specific loss of function effects against the gene of interest. Secondly, and actually the most common result when researching a novel gene, is no phenotype and no mis-targeting. Thirdly, specific loss of function effect against the gene of interest may occur, however this may be accompanied with a mis-

targeting phenotype. Lastly, the MO injection may result only in a mis-targeting phenotype (Ekker and Larson 2001).

In order to determine if any of the above are occurring, several different controls are necessary. A MO with reduced efficiency, due to 4 or 5 base pair mismatches to the experimental MO, will provide insight into any non-specific toxicity (Pickart *et al.* 2004). A more robust control to demonstrate MO specificity is to rescue the phenotype by co-injection of an mRNA that does not contain the MO target sequence. For example, with a translation blocking MO, the target sequence may be modified so as to not bind the MO while maintaining a KOZAC sequence, or in zebrafish the mammalian orthologue mRNA may be injected. This method is often limited by the fact that the temporal or spatial control of expression may be inappropriate, resulting in lack of rescue (Link and Megason 2008). Another approach is to phenocopy the first MO injections by injecting a second MO with non-overlapping sequence to the first. Likely the best approach in all cases to demonstrate specificity is to simultaneously target the same mRNA with two non-overlapping sequences. This synergy allows for each of the MOs to be injected at doses lower than when injected independently, thus decreasing the incidence of mis-targeting and non-specific effects (Pickart *et al.* 2004).

To date, MOs against *dnmt1* (Rai *et al.* 2006), *dnmt2* (Rai *et al.* 2007), and all of the *de novo* methyltransferases (Shimoda *et al.* 2005), have been injected into developing zebrafish embryos. In several instances insight has been gained into the function of methyltransferases in development, and, in two of these instances, they have been linked with terminal differentiation.

In a study examining the interplay between dnmt1 and a histone methyltransferase suv39h1, it was determined that terminal differentiation of the intestine, exocrine pancreas, and retina were impaired upon *dnmt1* MO injection, and partially restored with *suv39h1* overexpression (Rai *et al.* 2006). The authors concluded these epigenetic modifiers function in the same pathway. In the future, it will be very interesting to discover the specific genes that are under the control of the methyltransferases to result in this disrupted differentiation.

Until recently, the function of dnmt2 remained somewhat of an enigma. Mice lacking *Dnmt2* demonstrated no developmental phenotype, nor did they provide evidence of any changes in overall genomic DNA methylation levels (Goll and Bestor 2005), while overexpression in *Drosophila melanogaster* resulted in an increase in DNA methylation levels, though not necessarily at CpG sites (Kunert *et al.* 2003). Again, however, omission of *Dnmt2* in flies, did not result in any developmental abnormalities (Kunert *et al.* 2003). Finally, Dnmt2 is the most conserved of the methyltransferases, being present in lower eukaryotes such as *Schizosaccahromyces pombe*, which lacks Dnmt1 and Dnmt3 (Goll and Bestor 2005). Overall, there appears to be both support for and against Dnmt2 as a DNA methyltransferase (Rai *et al.* 2007). Zebrafish MO injections have helped provide insight into the controversy surrounding dnmt2 function. In a series of experiments with dnmt2 knockdown followed by rescue experiments with *dnmt2* mRNA that could only localize to the nucleus or the cytoplasm, Rai et al. (2007) demonstrated that proper terminal differentiation of the liver, retina, and brain was dependent on dnmt2 activity in the cytoplasm, but not the nucleus. This group further provided support that the RNA species targeted by dnmt2 was in fact tRNA (Rai *et al.* 2007).

Previously, zebrafish *de novo* DNA methyltransferases have been knocked down by MO injection (Shimoda *et al.* 2005). However, this study was focused on determining the methyltransferase responsible for methylating the *no tail* (*ntl*) gene during development. Shimoda *et al.* (2005) successfully demonstrated that following *dnmt7* MO injection, the CpG island of *ntl,* which normally displays methylation at nearly half of the CpG dinucleotides by 48 hpf in zebrafish, showed decreased levels of methylation to 20% of normal. This was the first demonstration of a *de novo* methyltransferase gene being directly implicated in gene-specific methylation during development. Interestingly, overall genomic DNA methylation levels remained unchanged. Most notably, no phenotype was associated with the disrupted methylation of *ntl*, though complete knockdown was not achieved, and some methylation remained at the *ntl* CpG island (Shimoda *et al.* 2005). In fact, no phenotypes were reported following MO injections against any of the *de novo dnmt*s (Shimoda *et al.* 2005).

Given the likelihood that the increased number of dnmt3s in zebrafish respective to mammals is a result of gene duplication, there may in fact be considerable redundancy in function among these proteins, and so we continued with our investigations, despite the report of lack of phenotype following *de novo* methyltransferase targeted MO injection.

To address questions regarding the role of the *de novo* DNA methyltransferases in vertebrate development, we injected MOs into 1-8 cell stage zebrafish embryos to knockdown expression of *dnmt3*, *dnmt5*, *dnmt6*, *dnmt7*, and *dnmt8* individually, followed by co-injections based on gene homology to each other, and in groupings based on homology to mammalian *Dnmt3a* and *Dnmt3b*. Co-injections therefore consisted of: *dnmt3* and *dnmt5* MOs; *dnmt3*, *dnmt5*, and *dnmt7* MOs; *dnmt6* and *dnmt8* MOs.

Injections against *dnmt4* will also be included once a working set of MOs has been obtained for the above mentioned genes, details to follow.

4.2 Methods

Wild type zebrafish were raised at 28°C by using standard methods (Westerfield 2000). Following injections, embryos were raised in Petri dishes containing embryo medium (Westerfield 2000) and were monitored for phenotype. Post-fertilization unfertilized embryos were removed from dishes between 4 and 6 hours.

Morpholino antisense oligonucleotides (MOs) were designed by and purchased from Gene-Tools LLC (Philomath, OR). MOs were dissolved to a stock concentration of 1mM and diluted with Danieau (58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO4, 0.6 mM Ca(NO3)2, 5.0 mM HEPES pH 7.6) prior to injection. Initial injections also included phenol red (final concentration 0.2%) for visualization. Unless stated otherwise, a minimum of 50 embryos were employed per injection dose, and data for each dose represent a minimum of three replicates. Injections were made into 1-8 cell stage embryos using a pressure injector (Nanoject II, Drummond Scientific Company. Broomall, PA). Pre-pulled needles (micro-tips, World Precision Instruments Ltd. Aston, Stevenage) were used to deliver 2.3 or 4.6 nl of morpholino. MOs of the following sequences were injected: *dnmt3* MO (5' CACATTTTCCTTTTCAACTATGAAT, Splice blocker); *dnmt5* MO (5' TGAACGTCTTCTAGCTGAAATAATA, Splice blocker); *dnmt6* MO (5' TGGTCCTCCATTGAGTTCATCACAG, Start site translation blocker); *dnmt6* mismatch MO containing five mismatches to *dnmt6* MO (5'-

TGcTCCTgCATTcAGTTgATCAgAG), 2nd *dnmt6* MO (5' ACACCAGTTTCGCCTTCTTCTCTGC, Start site translation blocker), 3rd *dnmt6* MO (5' CTCTGCCTGCAACGTAGGGAACAGC, Splice blocker); *dnmt7* MO (5' GTTCCAGACTAACATTTGTAGCCAT, Start site translation blocker), *dnmt7* mismatch MO (5'-GTTgCAGAgTAAgATTTcTAGCgAT); *dnmt8* MO (5' GTGTGTAAAAAGACACTTTCAAAAC, Start site translation blocker). Images were captured by using a Cannon Rebel XT digital camera mounted on a Motic SMZ168 dissecting microscope.

For mRNA injections, zebrafish dnmt6 (Genbank accession number AB196917) was amplified by RT-PCR using RNA reverse transcribed with M-MLV Reverse Transcriptase (Invitrogen Inc. Carlsbad, CA) according to the maufacturer's instructions and with 1 ug RNA and an Anc-T primer. PCR was carried out with modified primers to include 5 base pair changes to the translation start site region at the 5'end of the gene, but maintaining a KOZAK sequence (Forward primer 5' TCTACCATGGACACAATGGAAGACCA), and to incorporate a His-tag signal prior to a stop codon in the 3'end (Reverse primer 5' TTAGTGGTGATGGTGATGATGAGTCCGACGCAGGCGAA). The amplified product was cloned into pCR 2.1 vectors (TOPO TA cloning kit, Invitrogen Inc. Carlsbad, CA) and mRNA was synthesized (mMessage mMachine kit and Poly (A) Tailing kit, Applied Biosystems/Ambion. Foster City, CA) with the manufacturer's instructions.

For RT-PCR, embryos were collected at 10-12 hours, and in some instances 24 hours post fertilization. Collected embryos were either experimental (injected with splice

blocking MOs) or control (injected with Danieau). Total RNA was isolated using TRIzol reagent (Invitrogen Inc. Carlsbad, CA) with the manufacturer's instructions and cleaned using the RNeasy clean-up kit (Qiagen Inc. Valencia, CA) with the manufacturer's instructions. RT was carried out as described above and PCR was performed with primers spanning the exon to be excised (Figure 4.5). For *dnmt3* (AB196914) primers flank exon 5 with Forward Primer 1 (dnmt3F1 5'- GGTGGCCTTCATTAAACCTG) and Reverse Primer 1 (dnmt3R1 5'- ACATCGGATATGCTGCTTCC) producing an amplicon of 1710 bp, and the expected size of the amplicon upon excision of exon 5 is 211 bp. A second reverse primer, dnmt3R2 (5'- CTACGGATCTTAACGGTCTG), was designed to produce an amplicon of 2573 bp in the presence of exon 5, and 1074 bp in its absence (Figure 4.5). For *dnmt5* (AB196916) primers flanked exon 5 with Forward Primer 1 (dnmt5F1 5'- CCTGCTCAGAAACTCTGCTG) and Reverse Primer 1 (dnmt5R1 5'- TGGCTCAACGTCTTCCTCTT) producing an amplicon of 1717 bp, and the expected size of the amplicon upon excision of exon 5 is 248 bp. A second reverse primer, dnmt5R2 (5'- TCCCATTCACTGATCGTGGTGT), was designed to produce an amplicon 2377 bp, and 908 bp with the removal of the target exon (Figure 4.5). For *dnmt6* (AB196917) the exon to be removed contained the ATG translation start site. Primers spanning this exon were dnmt6F (Forward 5'- GCCAACATTCTGCAAGTCCT) and dnmt6R (Reverse 5'- GCCAAACCATGTAACCCATC), producing a transcript 707 bp, and an expected size of 356 bp upon excision of the exon.

For western blotting, embryos were collected at 12 and 24 hours post fertilization, and homogenized in SDS sample buffer (Westerfield 2000). Samples were boiled for 4 minutes and supernatants were collected following 5 minutes centrifugation. Protein was

separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to polyvinylidene difloride membrane. Presence of His-tag protein was determined by hybridization with primary antibody mouse anti-His (Zymed Laboratories, Invitrogen Inc. Carlsbad, CA), and secondary antibody horseradish peroxidase (HRP)-goat anti-mouse IgG (Zymed Laboratories, Invitrogen Inc. Carlsbad, CA), followed by 3,3'-Diaminobenzidine (DAB) visualization (Roche Ltd. Mississauga, ON) with the manufacturer's instructions.

4.3 Results and Discussion

Dnmt3, dnmt5, and dnmt7 knockdown

To determine *dnmt3* and *dnmt5* methyltransferase function in zebrafish development, we injected splice-blocking antisense MOs into 1-8 cell stage zebrafish embryos. These two genes are most similar to mammalian *Dnmt3b*, and likely represent a tandem gene duplication (Smith *et al.* 2005). Given dnmt3 and dnmt5 are 72% similar at the amino acid level, the possibility of functional redundancy is quite high, and therefore co-injections were carried out. However, *dnmt7* as well as *dnmt4* which shows highest homology of the zebrafish *dnmts* to mammalian *Dnmt3b* are also present, and co-injections of more than two gene MOs were also undertaken.

Both the *dnmt3* and *dnmt5* MOs were designed to excise an exon in the 5' region of the respective genes. In both instances, the exons to be excluded were not divisible by three, and therefore resulted in nucleotide frameshifts when not included in the transcripts, likely producing nonsense products (Figure 4.5).

Embryos injected with 1 ng, 5 ng, and 10 ng of either *dnmt3* or *dnmt5* MO independently did not demonstrate any observable penetrant phenotype. As well, embryos receiving co-injections of 3 ng of each *dnmt3* MO and *dnmt5* MO, and embryos receiving 6 ng of each MO, did not demonstrate any abnormalities in development. In order to determine whether these results indicated a lack of observable relevance for dnmt3 and dnmt5 in development, or whether the MOs were ineffective, embryos injected with 10 ng of each MO in single MO injections, and a co-injection of both MOs (6 ng each) were collected at 12 hpf. Presence of disrupted splicing was determined by RT-PCR (Figure 4.6). Although the splice blocking MOs would not alter maternally supplied transcripts, we would expect to be able to observe altered splicing of zygotic transcripts by RT-PCR. Unfortunately, *dnmt3* MO did not result in removal of the large exon, and *dnmt5* MO appears at this stage to be only moderately effective in targeting the transcript of interest and resulting in the large exon excision. Given that, for splice blocking injections, a greater amount of MO must be injected in order to target transcripts during the narrow window before they are properly edited following transcription, single injections of *dnmt3* MO and *dnmt5* MO were carried out with 15 ng and 30 ng of the respective MOs. Embryos collected at 6 hours, followed by RT-PCR with extracted RNA, indicate that even at these very high dose injections there was no evidence of exon excision upon *dnmt3* MO injection, and only mild efficacy of exon excision occurring with *dnmt5* MO injections (Figure 4.7).

Treble injections were carried out with *dnmt3*, *dnmt5*, and *dnmt7* MOs alongside above mentioned injections prior to determining lack or moderate efficacy of *dnmt3* and *dnmt5* morpholinos. As would be expected given the RT-PCR results from *dnmt3* and

dnmt5 injections, no phenotype was observed in the developing embryos. However, this treble injection is a good example of the types of injections that may be performed in order to begin teasing apart function of the various zebrafish *dnmts*. Once working MOs for *dnmt3* and *dnmt5* are obtained, functional overlap from all three genes may be determined by injecting lower doses of each of the MOs. Also, for instance, a co-injection of *dnmt3* and *dnmt7* MOs may provide insight into the function of the CH-domain found in these two genes, and highlight specific roles of *dnmt3* beyond any overlapping function with *dnmt5*. This is not a domain typically found in DNA methyltransferases, nor in any of the other zebrafish *dnmt* genes, and its function here is not known. Although there was no phenotype in dnmt7 knockdown embryos, this MO was demonstrated to alter methylation at at least one gene (Shimoda *et al.* 2005), and perhaps when co-injected with *dnmt3* MO, observable developmental defects may result.

dnmt6 and dnmt8 gene knockdown

Another very logical co-injection based on similarity to each other and potential overlap in function is to knockdown dnmt6 and dnmt8, which show the greatest similarity to mammalian Dnmt3a (amino acid identity 81% and 76% respectively), and which are 70% similar to each other at the amino acid level. Studies in mice have suggested that Dnmt3a may be most relevant for gene specific methylation, and thereby lineage determination. The importance of Dnmt3a is underlined by the lethality that occurs shortly after birth in mice lacking Dnmt3a. Additionally, of the six *de novo* methyltransferases in zebrafish, *dnmt6* and *dnmt8* are the only two that show high

similarity to *Dnmt3a*, as the others are more similar to *Dnmt3b*, simplifying the number of genes requiring targeting.

Prior to performing co-injection experiments, a morphant phenotype was observed based on *dnmt6* MO injections alone. Injections of a relatively low dose of dnmt6 MO (3 ng or less) produced results that were in agreement with the findings of Shimoda *et al.* (2005) which indicated a lack of phenotype (Table 1). However, we observed several developmental abnormalities when MO doses were increased to 5-10 ng injections (Figure 4.8).

Firstly, a dose response was carried out to determine a range of phenotypes (Table 4.1). Although all ranges of phenotype were detected at each of the doses, a higher frequency of less severe or more severe phenotypes corresponded with lower dose and higher dose injections, respectively. Penetrance of phenotype increased with increasing dose (Table 1).

Less severe morphants demonstrate one kink, or several subtle kinks, in the anterior posterior axis (Figure 4.8). With this less severe phenotype, fry 72 hpf and older are capable of swimming in a more or less straight line. More severe morphants demonstrate a lateral curvature, so that the embryos take a U-shape, and fry are incapable of swimming in a straight line, moving only in circles when attempting to swim. As well, undulations in the notochord were particularly pronounced (Figure 4.9 and Figure 4.10). At very high doses of dnmt6 MO (10 ng) an increased incidence of embryos demonstrating non-specific necrosis occur, also resulting in a higher death rate, but as Figure 4.11 illustrates this is not true in all cases. In many instances, these more severe morphants with very severely disrupted notochords do not demonstrate overall necrosis,

nor do the notochord abnormalities resemble those typical of non-specific effect. At these high doses, there is some reduction in head size, and abnormal eye development; again, however, these do not appear entirely similar to what is observed in p53 mediated non-specific effects. Notochord undulations are also accompanied by pericardial edema, and often absence of either one or both pectoral fins, and reduced tail length (Figure 4.11 and Figure 4.12). Whether some of these effects are non-specific may be determined by identifying a second functional *dnmt6* MO, and lower dose co- injections may provide further insight.

Control injections with *dnmt6* mismatch MO (same sequence as *dnmt6* MO with 5 base pair mismatches) did not result in any observable phenotypes, even when carried out at similarly high doses (Table 4.1 and Figure 4.13). This demonstrates that this phenotype is not due to toxicity from the presence of any MO, the process of injection, or the buffers used and supports that it is a direct result of dnmt6 removal.

In order to demonstrate specificity of the *dnmt6* MO, duplication of phenotype has been attempted with two additional different MOs designed against *dnmt6* transcript, but with non-overlapping sequence to the first MO. Phenocopying the morphants has proven difficult given one attempted translation blocking morpholino (2^{nd} *dnmt6* MO) causes embryonic death at low doses, and severe necrosis, typical of off-target effects (Figure 4.14), while another splice blocking MO (3^{rd} dnmt6) was not effective and did not result in splicing verified by RT-PCR (Figure 4.15).

Regardless of the high toxicity of the 2^{nd} *dnmt6* translation blocking MO, co-injections of *dnmt6* MO and 2^{nd} *dnmt6* MO were carried out at levels that do not incur highly penetrant phenotype with *dnmt6* MO (5 ng) and levels that are low toxicity in 2^{nd}

dnmt6 MO injections (2 ng) in order to determine if the 2nd *dnmt6* MO potentiated the disrupted notochord phenotype. In this instance, the incidence of kinked or disrupted notochord was not increased above levels that would be expected from 5 ng alone of *dnmt6* MO (Table 4.2). This indicates that either one of the MOs is not targeting dnmt6, or even possibly, that neither MOs are on target. Clearly, injections of the 2nd *dnmt6* accompanied with a p53 MO may be carried out in order to determine if there is presence of phenotype (*i.e.* notochord disruption) that is being masked by the high degree of necrosis through the trunk region and head that is caused by injections with this MO above 2 ng.

Co-injections were carried out to determine knockdown specificity with *dnmt6* MO and a His-tagged mRNA that had a modified ATG site so as to not complementary base pair with the *dnmt6* MO, but still maintain a Kozak sequence. Future rescue attempts may also consider designing the mRNA so that the amino acid sequence is maintained, and changes to mRNA are based on the degenerate code.

Although there does appear to be some amelioration of phenotype with the rescue injections (29% showing phenotype after rescue vs. 44% without rescue), this difference was not statistically significant (χ^2=1.459, *df*=1, 0.25>*p*>0.10, α=0.05) and cannot satisfactorily determine *dnmt6* MO as specific (Table 4.3). Western blots carried out with protein extracted from 12 and 24 hpf injected embryos and anti-His antibody demonstrated no presence of the His-tag. This indicates that the modified injected mRNA did not result in dnmt6 protein levels that may be detected by western blot, and/or possibly the transcript is quickly degraded following injection (Figure 4.16). Another possibility is that, despite efforts to de-yolk embryos prior to collection for western

blotting, high levels of yolk protein were still apparent when performing western blots, making it difficult to load enough embryo specific protein to facilitate signal detection. Additionally, we were unable to demonstrate efficacy of knockdown of native dnmt6 by western blotting given the lack of cross-reactivity of tested antibodies to zebrafish dnmt6.

Although the phenotype that was obtained with these injections is not typical of non-specific effects and is not due to general toxicity of the MO or injection technique, our inability to identify an effective second MO, effectively rescue the phenotype or monitor protein levels in our morphants allows for the possibility that the phenotype generated was either partially or solely due to non specific or mis-targeting effects.

Co-injections were also carried out with *dnmt6* MO and an ATG start site blocking MO against *dnmt8*. This experiment, if resulting in a phenotype, would not only provide evidence for overlap between dnmt6 and dnmt8, but would also offer some support for the *dnmt6* MO as specific. However, the designed *dnmt8* MO also results in high levels of necrosis, reduced embryo size, abnormal trunk and tail development and embryonic death at low doses. This MO is also therefore a candidate for carrying out co-injections with a p53 MO that may allow for a specific phenotype to be determined without the complication of overall necrosis, reduced embryo size, and curled tail. Although additional *dnmt8* MOs may be designed in the 5'UTR, splice blocking MOs targeting *dnmt8* are not possible at this time given the low sequence quality available for this gene at the genomic level.

The notochord has a dual role as it serves not only as the axial skeleton to the developing embryo until other elements develop, such as the vertebrae, but is also a source of midline signals that pattern surrounding tissues (Stemple 2005). Notochord

tissue is most closely related to cartilage. As such, in higher vertebrates, this tissue also becomes ossified where vertebrae form, while intervertebral areas contribute to the nucleus pulposis of vertebral discs (Stemple 2005). With respect to its function as a source of signalling to surrounding tissues during development, the notochord is involved in specifying ventral fates in the central nervous system, controlling some aspects of left-right asymmetry, inducing pancreatic fates, determining venous versus arterial identity of major axial blood vessels, specification of the cardiac field, as well as specifying a variety of cell types in forming somites (Stemple 2005). Several of the additional phenotypes observed in *dnmt6* MO injections may therefore be in keeping with an improperly differentiated notochord, incapable of carrying out normal signalling functions.

Notochord cells are specified during gastrulation, arising in the shield region. The first major transition in notochord development is the formation of molecularly and morphologically distinct chordamesoderm (Stemple 2005). Between 6 and 12 hpf, through a number of cell movements, the chordamesoderm cells come to lie at the dorsal midline of the embryo (Figure 4.17 and Figure 4.18) (Stemple 2005; Solnica-Krezel 2006). Starting around 20 hpf, the chordamesoderm cells begin to differentiate to mature notochord, as cells secrete a thick extracellular sheath, and they also acquire vacuoles which provide hydrostatic pressure that, in combination with the sheath, offer the rod-like structure of the notochord (Figure 4.19) (Stemple 2005). For proper transition from chordamesoderm to mature notochord, down-regulation of genes involved in early development is required, as well an increase in mRNA for genes involved in mature notochord formation. Without down-regulation of certain regulatory and structural genes,

notochord differentiation may become arrested (Hawkins *et al.* 2008). Notochord cells continue vacuolation and differentiation until 36 hpf (Anderson *et al.* 2007).

A number of identified mutants have already provided much insight into several loci that are required for notochord development (Figure 4.18). Based on phenotypic analysis, mutations affecting notochord development may be placed into several categories according to the stage of development affected, and further grouped based on the specifics of the phenotype (Stemple *et al.* 1996). Some of these groupings include no chordamesoderm formation, failure of notochord cells to differentiate and become vacuolated, shortened notochord, or the category that seems most similar to *dnmt6* morphants, notochords that are folded or undulate (Stemple *et al.* 1996).

As one might imagine, in instances where the notochord fails to be specified, lack of signalling results in very severe defects for the embryo, as is seen in mutants of the *floating head* (*flh*) locus (Figure 4.20). Clearly, mutations affecting notochord development have profound effects on body patterning (Anderson *et al.* 2007). Although several loci have been found to be relevant for signalling, the majority have been identified as being important for structural aspects of notochord function (Stemple 2005). For example, in the category of mutants where notochord differentiation is disrupted, as with mutants *bashful*, *grumpy*, *sleepy*, *sneezy*, *happy*, and *dopey*, phenotypically, embryos are often shortened, and at the molecular level, secretion by the cells or notochord sheath components are defective (Figure 4.21).

While the abovementioned mutants have been characterized at the molecular level, the group most similar to *dnmt6* morphants, demonstrating well-differentiated notochords, but that are distorted in dorsomedial and/or mediolateral planes, have not

been so characterized (Anderson *et al.* 2007). This group of mutants includes *levianthan*, *gulliver*, *crash test dummy*, *zickzack*, *quasimodo*, *kinks*, and *wavy tail* (Figure 4.22) (Odenthal *et al.* 1996; Stemple *et al.* 1996). It is difficult to categorize this group as to when they may be having an effect during notochord development, as the phenotypes could be arising for a number of potential reasons (Odenthal *et al.* 1996). For instance, these loci may control early events such as general aspects of cell movement during gastrulation and proper convergence and extension of the chordamesoderm. With morphogenesis of the tail affected, notochord undulations may result from a lack of space, if the tail is shortened (Stemple *et al.* 1996). Alternatively, these loci may be very relevant for the transition from chordamesoderm to mature notochord and interactions with the surrounding tissues. Very possibly, this phenotype could also result from improper adhesion to the notochord sheath or an increase in cell division in the notochord. The undulating notochord of *dnmt6* morphants may be further explored once evidence for knockdown specificity has been gained.

4.4 Conclusion

Due to some evidence of functional overlap between Dnmt3a and Dnmt3b in mammals, along with the potential redundancy in the number of *dnmt3s* present in zebrafish, a number of combinations of co-injections will no doubt provide new insights into the *de novo* DNA methyltransferases, their overlap, and their specific functions. Imperative to this work will be the development of specific, efficient MOs against each of the transcripts.

At the outset, we had imagined co-injections with MOs would result in developmental abnormalities, and that these embryos may then be collected for microarray analysis. Although we present here only one instance where phenotype was observed following MO injection, we have yet to perform co-injections with effective specific MOs. Once a set of MOs is obtained for each of the *de novo* methyltransferases, and these are followed with microarray analysis, we will then begin to be able to determine genes that are candidates for being directly under the control of methylation, or at least downstream of genes regulated by methylation. Based on the different co- or triple injections *etc.*, we can begin to tease apart which genes may be under the control of several of the dnmt3s, and which genes are under specific regulation by only one of the dnmt3s. Of course bi-sulfite sequencing of the promoter regions of the genes of interest will provide direct evidence for disrupted methylation following injection, and ultimately provide a set of genes that may be used to study many of the remaining unanswered questions regarding dnmt3 targeting, and how the epigenetic layers are involved in programming embryonic development.

4.5 Literature Cited

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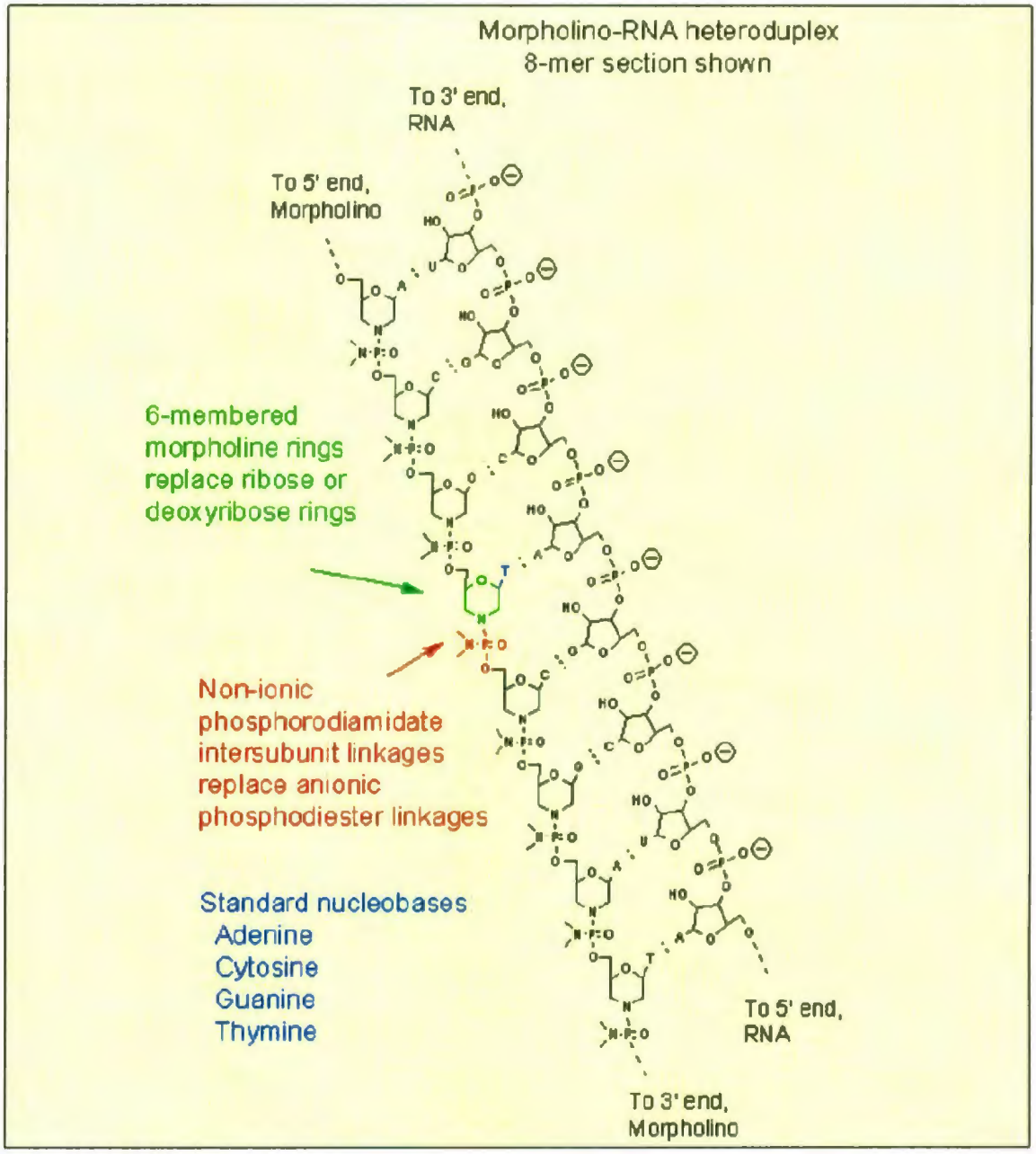

Figure 4.1 Schematic of a MO oligonucleotide complimentary base pairing to mRNA. MOs are synthesized to target specific transcripts of interest, and the backbone of the MO oligonucleotide is unrecognizable to cellular enzymes and is not degraded (with permission from Jon D. Moulton, Gene Tools, LLC).

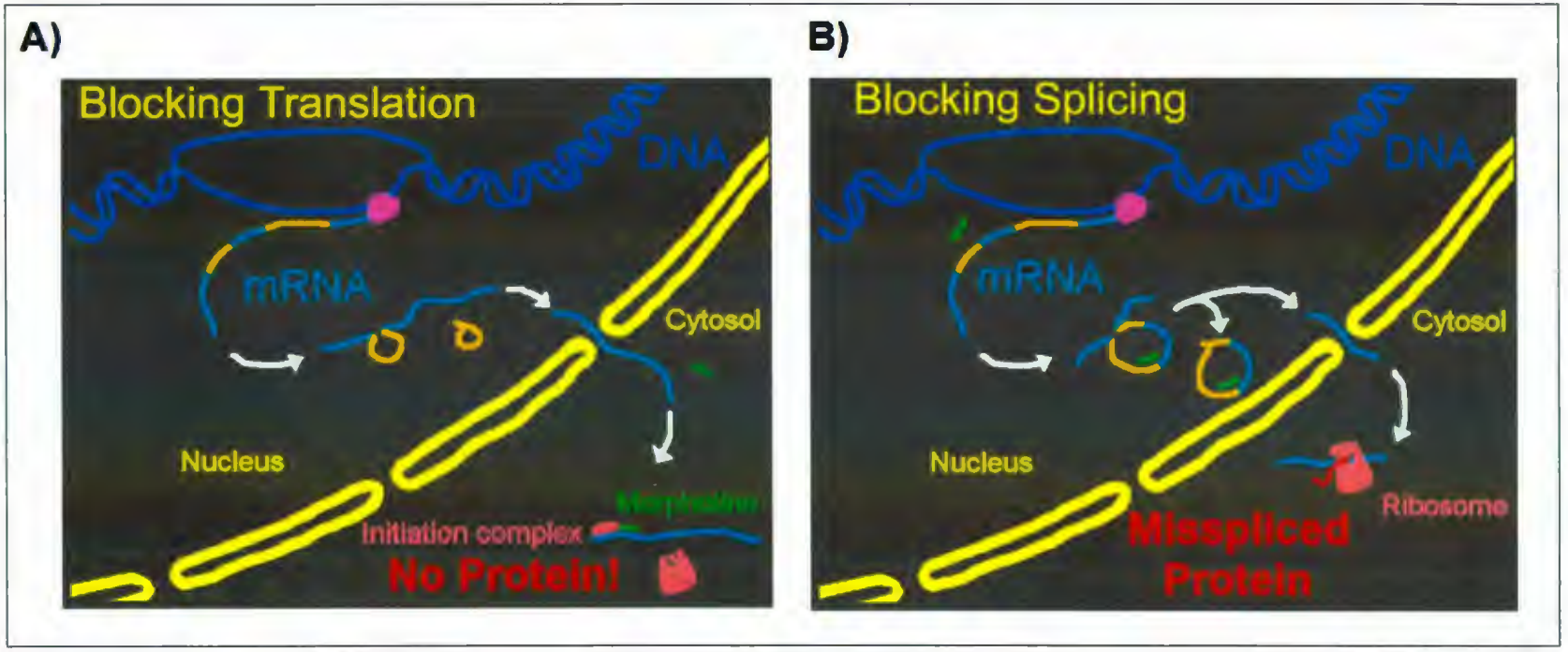

Figure 4.2 Schematic of mechanisms by which MOs disrupt gene function. A) MOs are targeted to the 5'UTR of the mRNA and prevent progression of the initiation complex. B) A pre-mRNA splice-site is targeted resulting in a mispliced protein (with permission from Jon Moulton, GeneTools, LLC).

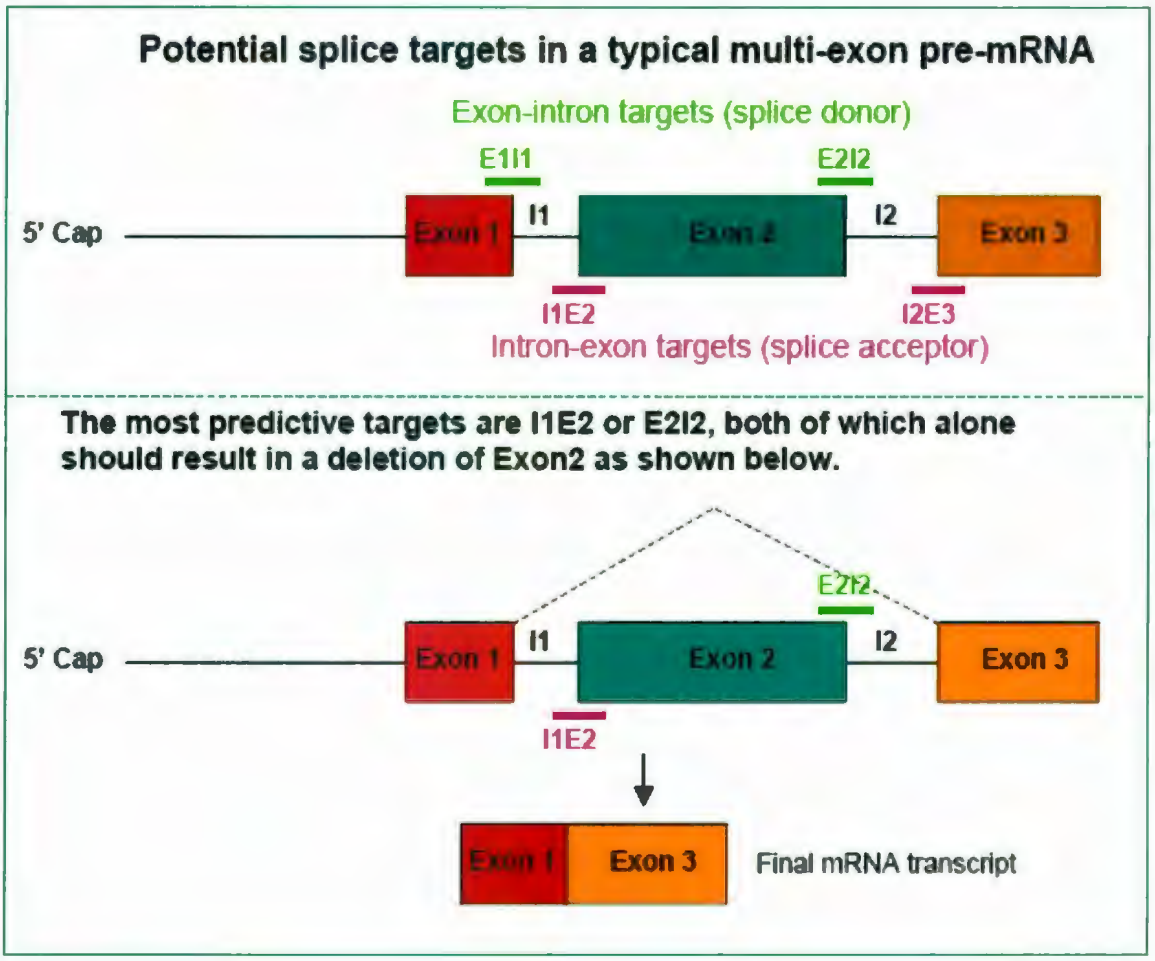

Figure 4.3 MOs may block nuclear processing. Exon-intron or intron-exon boundaries may be targeted, resulting in an exon excision, or intron inclusion. Here exon 2 is excised, and the product may be characterized by RT-PCR (adapted from Gene Tools, LLC. www.gene-tools.com).

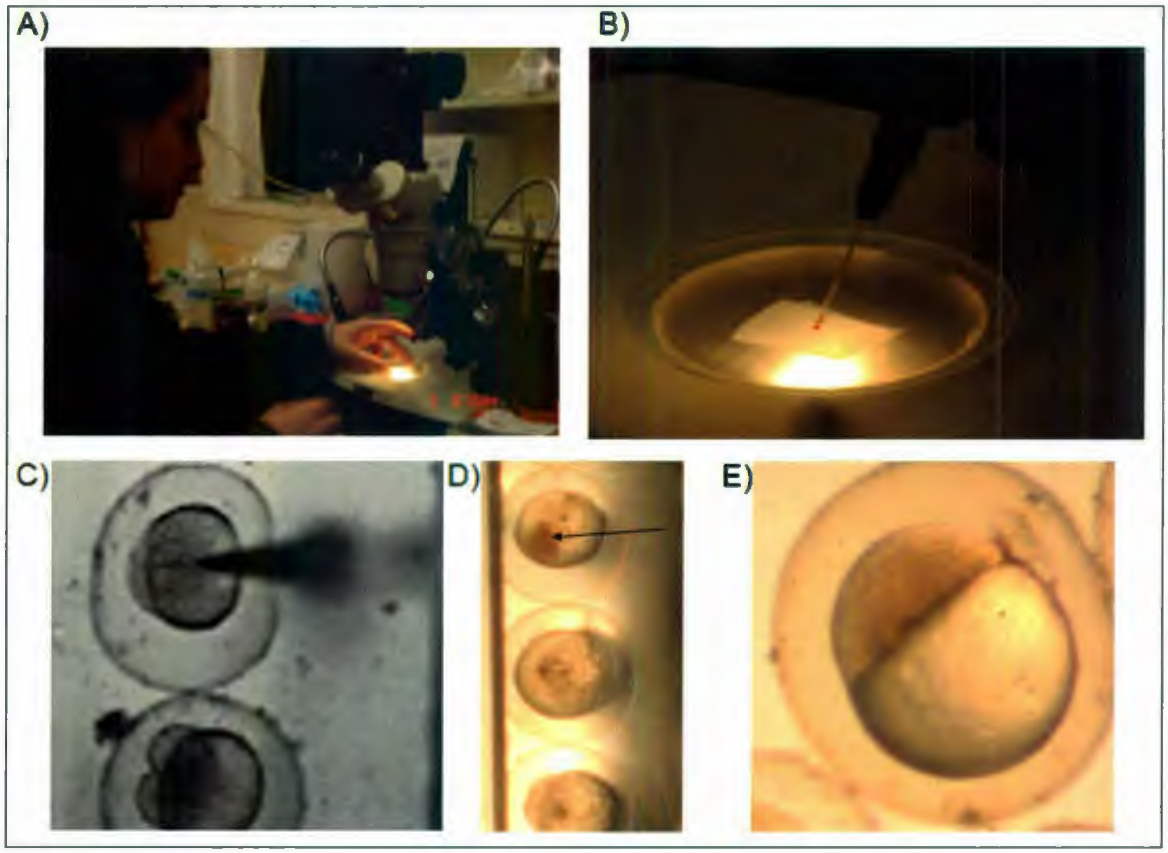

Figure 4.4 Morpholinos are microinjected into embryos. A) Injections are performed using a microinjector, a micromanipulator, and dissecting microscope. B) Loading the needle with phenol red dyed morpholino. C) The needle punctures through the chorion of an embryo and MO is delivered into the yolk of the developing embryo. D) When phenol red is used, a red spot may be observed when injection is properly performed. E) Embryos are raised in embryo medium and observed for phenotype.

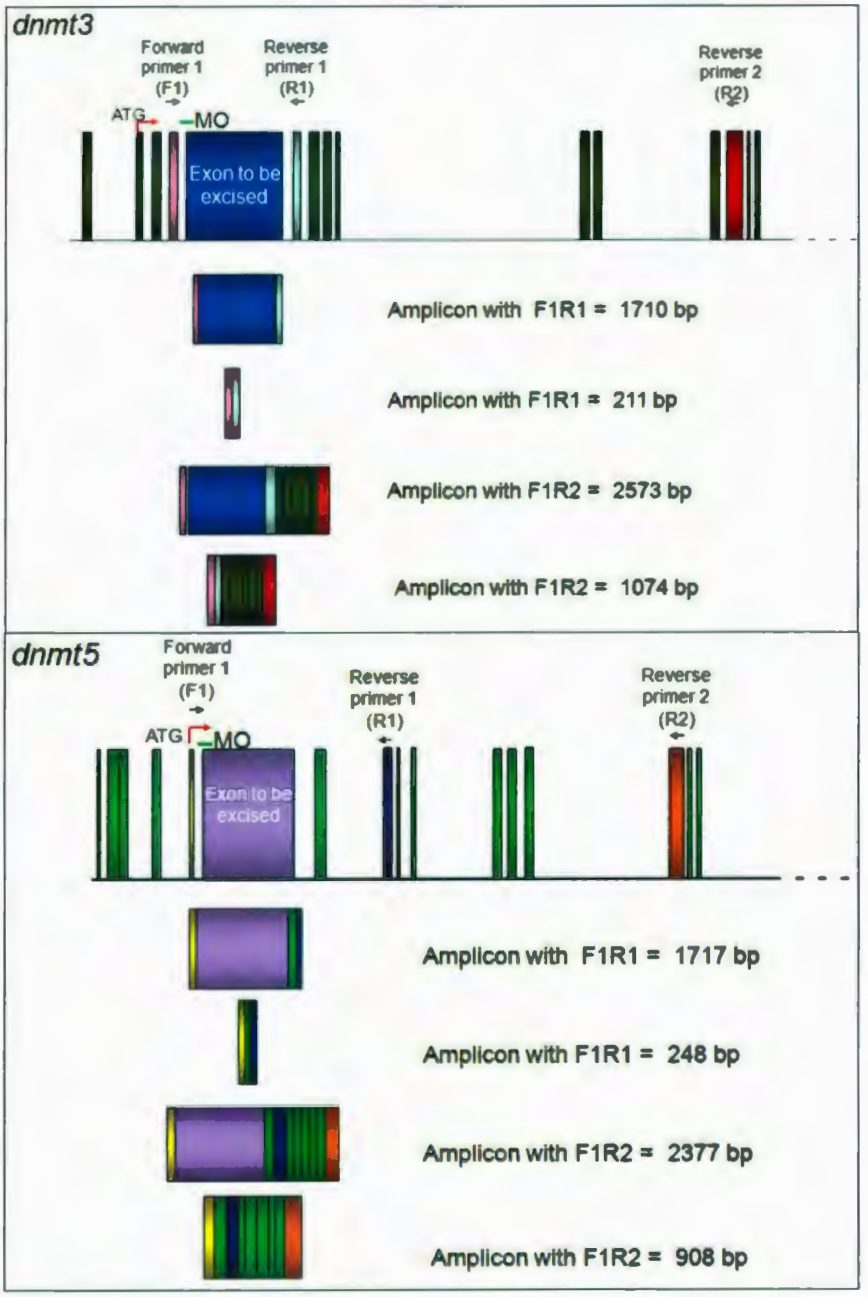

Figure 4.5 Schematic of *dnmt3* and *dnmt5* 5' genomic structure. The exons to be excised in the presence of targeting MOs are indicated, as well as the flanking primers used to characterize the RT-PCR products. Schematic of amplicons are represented below the genomic structure (Coloured bars represent exons, black line between coloured bars represent introns).

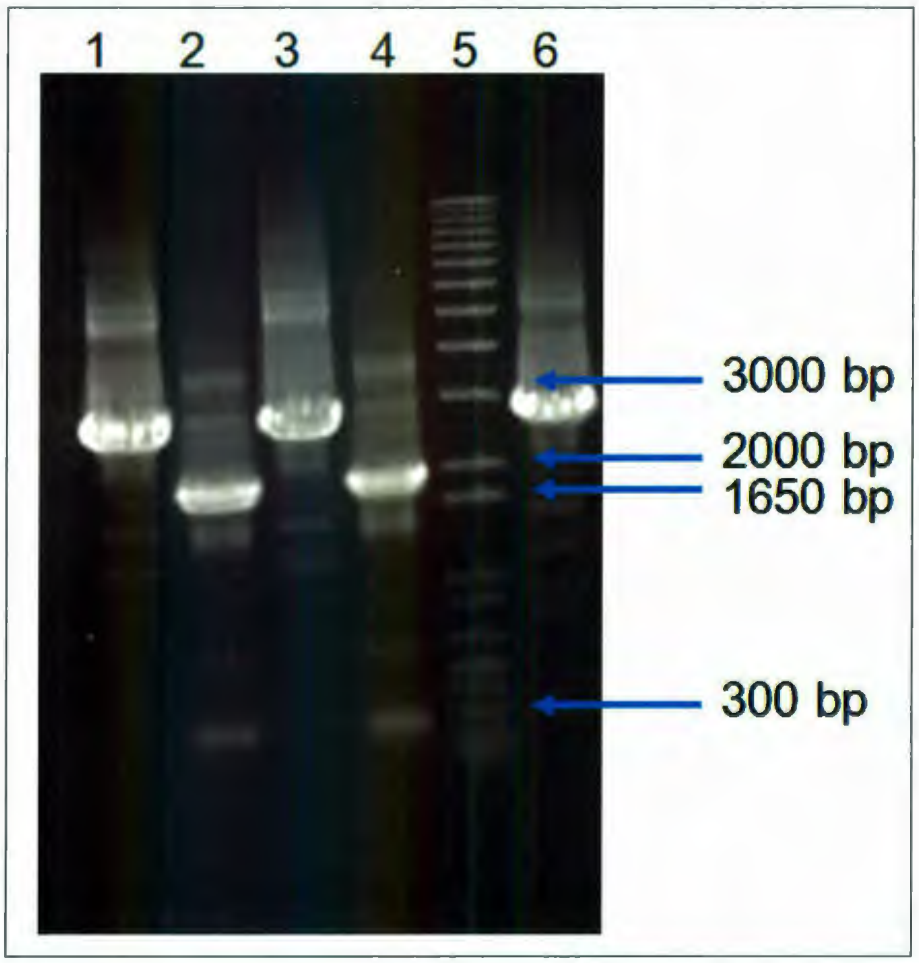

Figure 4.6 MO injected and control embryos were collected at 10-11 hpf, RNA was extracted, and RT-PCR was performed. **Lane 1**: 10 ng *dnmt3* MO injected embryos. RT-PCR with dnmt3F1R2 primers. Expected amplicon 2573 bp, and if exon excised 1074 bp. **Lane 2**: 10 ng *dnmt5* MO and RT-PCR with dnmt5F1R1. Expected amplicon 1717 bp, and if exon excised 248 bp. **Lane 3:** Embryos co-injected with dnmt3 and dnmt5 (6 ng each). RT-PCR primers dnmt3F1R2. **Lane 4:** Embryos co-injected with dnmt3 and dnmt5 (6 ng each). RT-PCR primers dnmt5 F1R1. **Lane 5:** 1 kb Plus DNA ladder. **Lane 6.** Danieau control injected embryos. RT-PCR primers dnmt3F1R2.

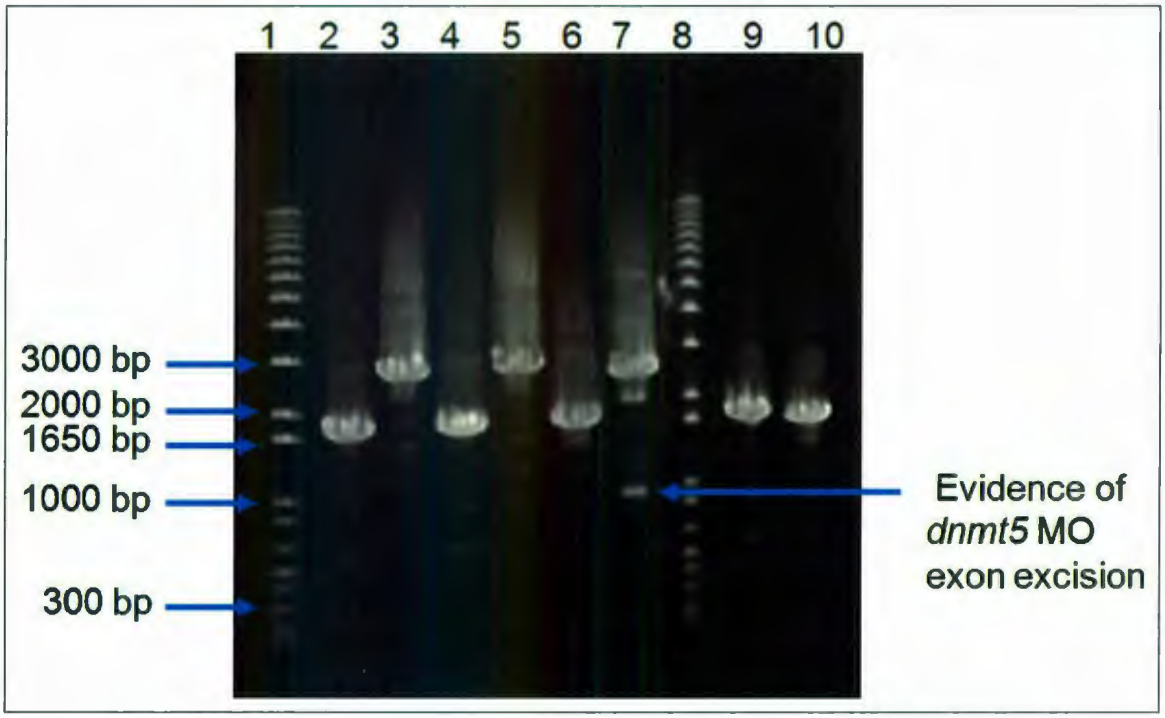

Figure 4.7 MO injected and control embryos were collected at 5-6 hpf, RNA was extracted and RT-PCR was performed. **Lane 1:** 1 Kb Plus DNA Ladder. **Lane 2**: 15 ng *dnmt3* MO and RT-PCR with dnmt3F1R1 primers. Expected amplicon 1710, and if exon excised 211 bp. **Lane 3**. 15 ng *dnmt3* MO with dnmt3F1R2 primers. Expected amplicon 2573 bp, and if exon excised 1074 bp. **Lane 4.** 30 ng *dnmt3* MO with dnmt3F1R1primers. **Lane 5.** 30 ng *dnmt3* with dnmt3F1R2 primers. **Lane 6:** 30 ng *dnmt5* MO and RT-PCR with dnmt5F1R1 primers. Expected amplicon 1717 bp, and if exon excised 248 bp. **Lane 7**: 30 ng *dnmt5* MO with dnmt5F1R2 primers Expected amplicon is 2377 bp, and if exon excised 908 bp. Fainter band present indicated some MO efficacy for dnmt5. **Lane 8:** 1Kb Plus DNA ladder. **Lane 9:** Uninjected control embryos. Primers dnmt3F1R1. **Lane 10**: Uninjected control embryos. Primers dnmt5F1R1.

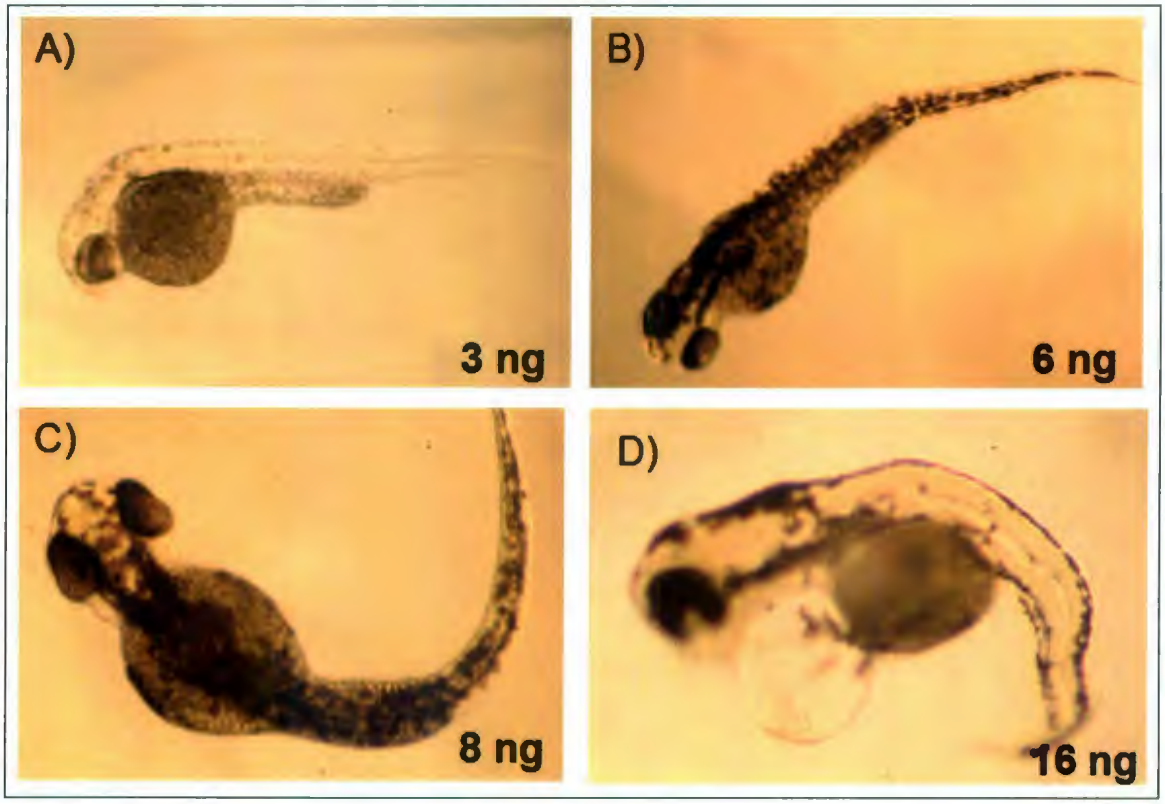

Figure 4.8 Embryo phenotype resulting from increased doses of *dnmt6* MO. A) Embryos injected with 3 ng or less do not demonstrate any phenotype. B) An embryo demonstrating several subtle kinks or undulations of the tail and notochord (notochord not visible here). C) At increased doses, a greater number of embryos display this U-shape lateral curvature and are incapable of swimming in a straight line. Often embryos will also have pericardial edema and pronounced undulations of the notochord. D) Very high doses of *dnmt6* MO resulted in a shortening of the body, multiple notochord undulations, severe pericardial edema, no fins, and disrupted eye development. Some of this severe phenotype may result from non-specific effects.

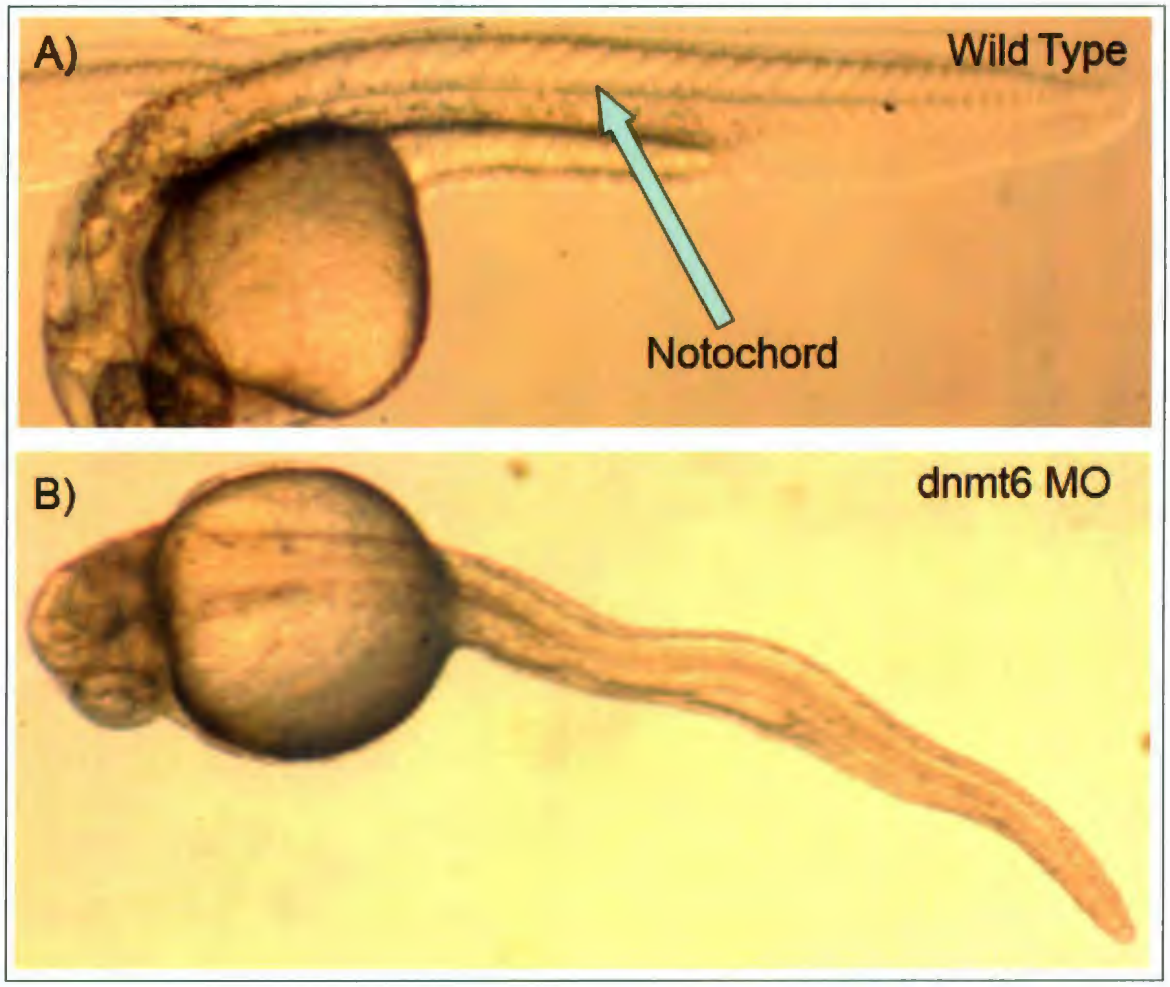

Figure 4.9 A) Photo of wild-type 24 hour embryo demonstrating notochord structure. B) Undulations in the notochord and tail of *dnmt6* MO injected embryos (8 ng MO).

Figure 4.10 Undulations in the tails of zebrafish fry and the kinks in the notochord are very evident at later stages (seen here 6 dpf 10 ng dnmt6 MO injected embryos). Also evident is the lack of pectoral fins on the lower fry.

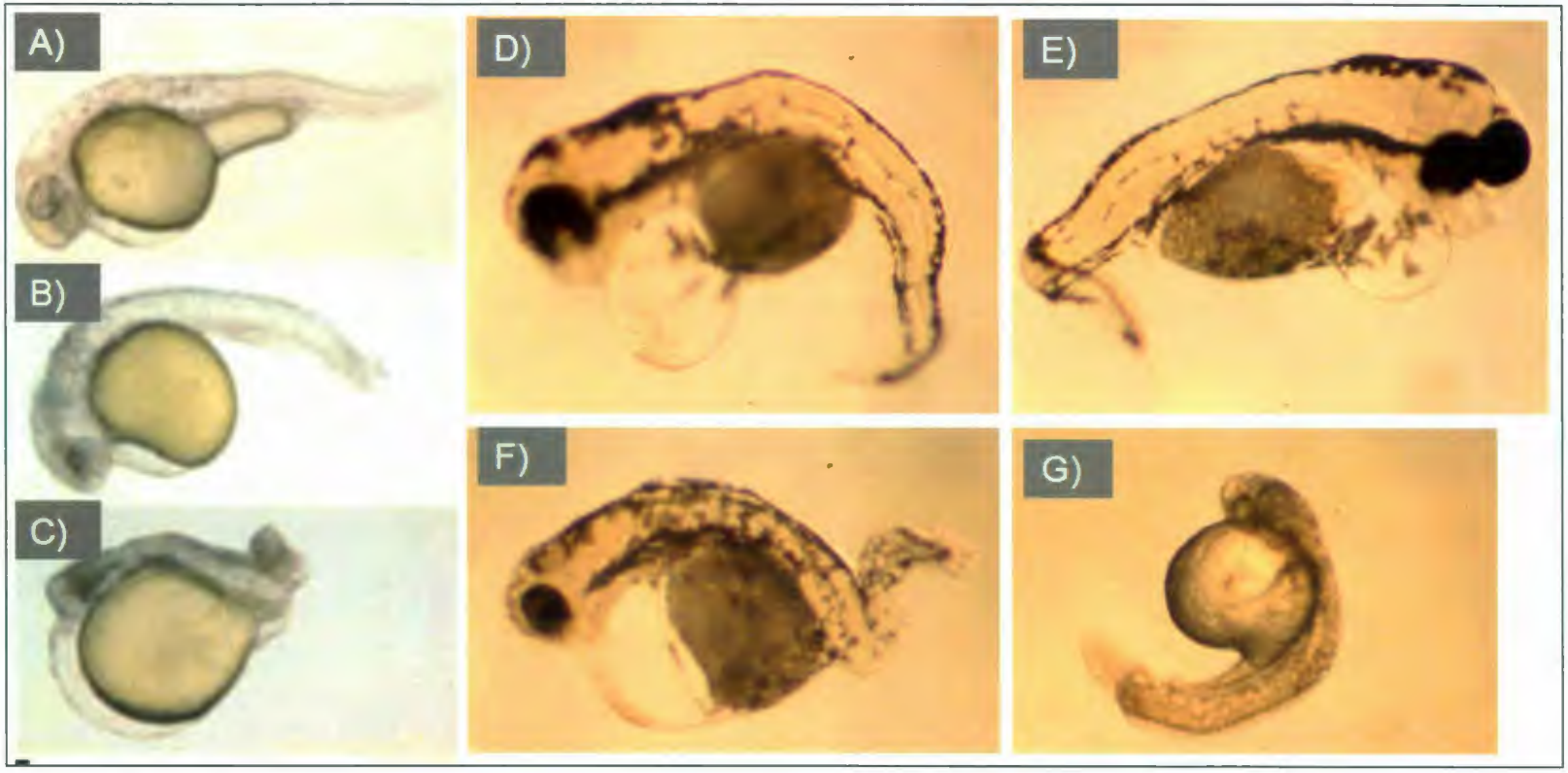

Figure 4.11 Comparison of embryos demonstrating low (A), intermediate (B), and severe cell death (C) phenotype mediated through p53 activation at 30 hpf with D-G) embryos receiving 16 ng *dnmt6* MO injections. Cell death is observed as highly dense opaque areas. Here, embryos F) and G) more closely resemble phenotypes seen in B) and C) with necrosis evident in tail and head regions. Structures in D) in E) on the other hand are more easily distinguished and appear to be simply more severe than phenotypes observed at lower *dnmt6* MO injections (A, B, and C adapted from Urtishak *et al.* 2003).

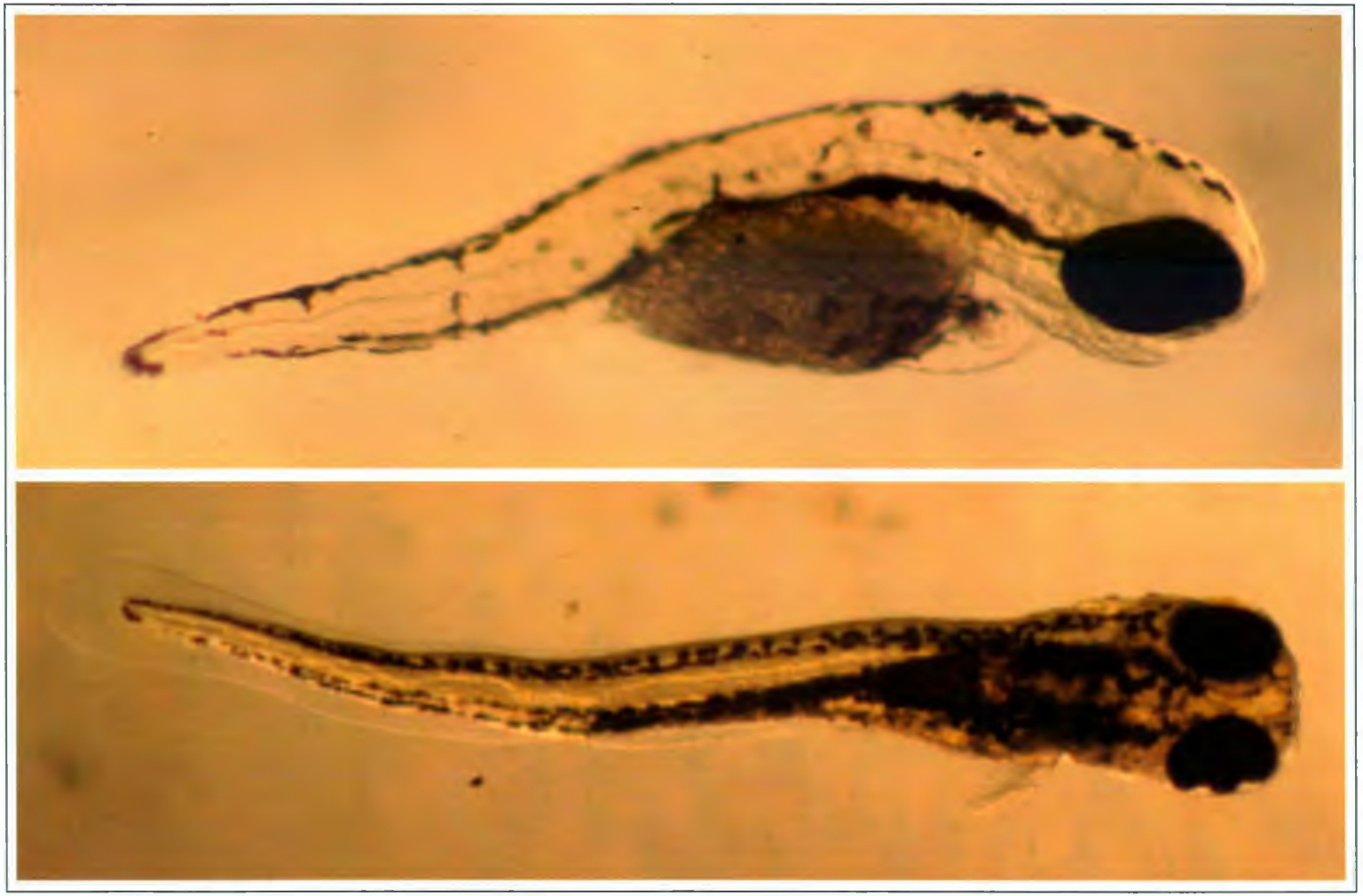

Figure 4.12 *dnmt6* MO injected 72 hour embryos demonstrating lack of fins (above) or lack of one pectoral fin (below). Also apparent is the characteristic disrupted notochord structure (both) and some pericardial edema (above).

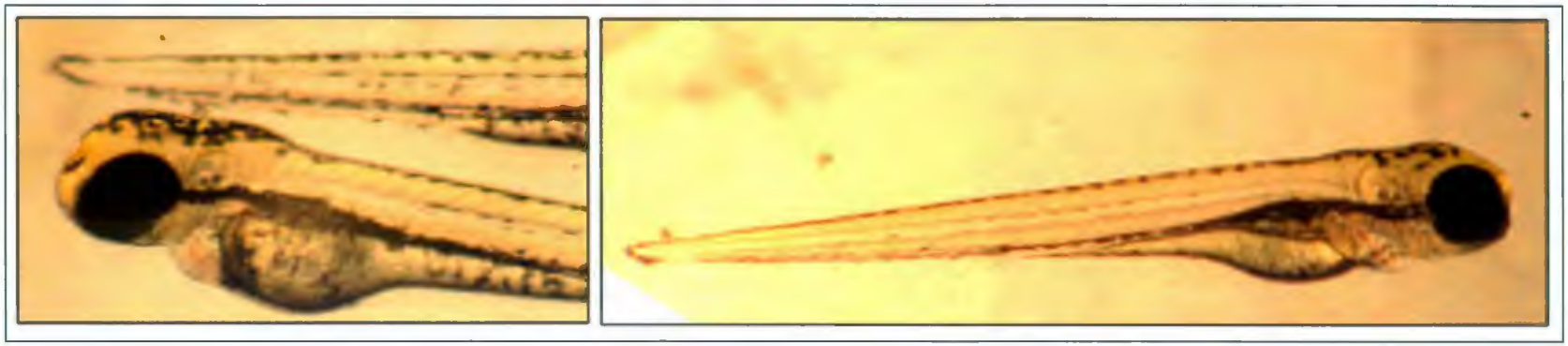

Figure 4.13 Control injections with 10 ng dnmt6 mismatch MO. 72 hour hpf embryo seen here, no observable phenotype.

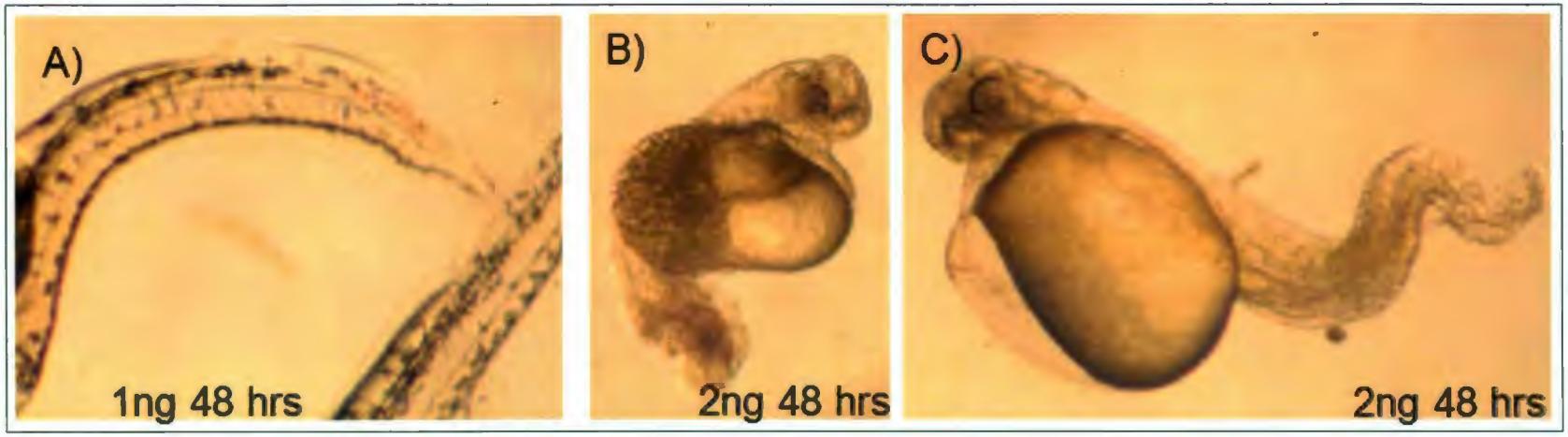

Figure 4.14 Severe cell death phenotype following 2nd *dnmt6* MO injection. In a couple of instances, phenotype duplication was observed (A), however, most typical results for injections with this MO are seen in B) and C).

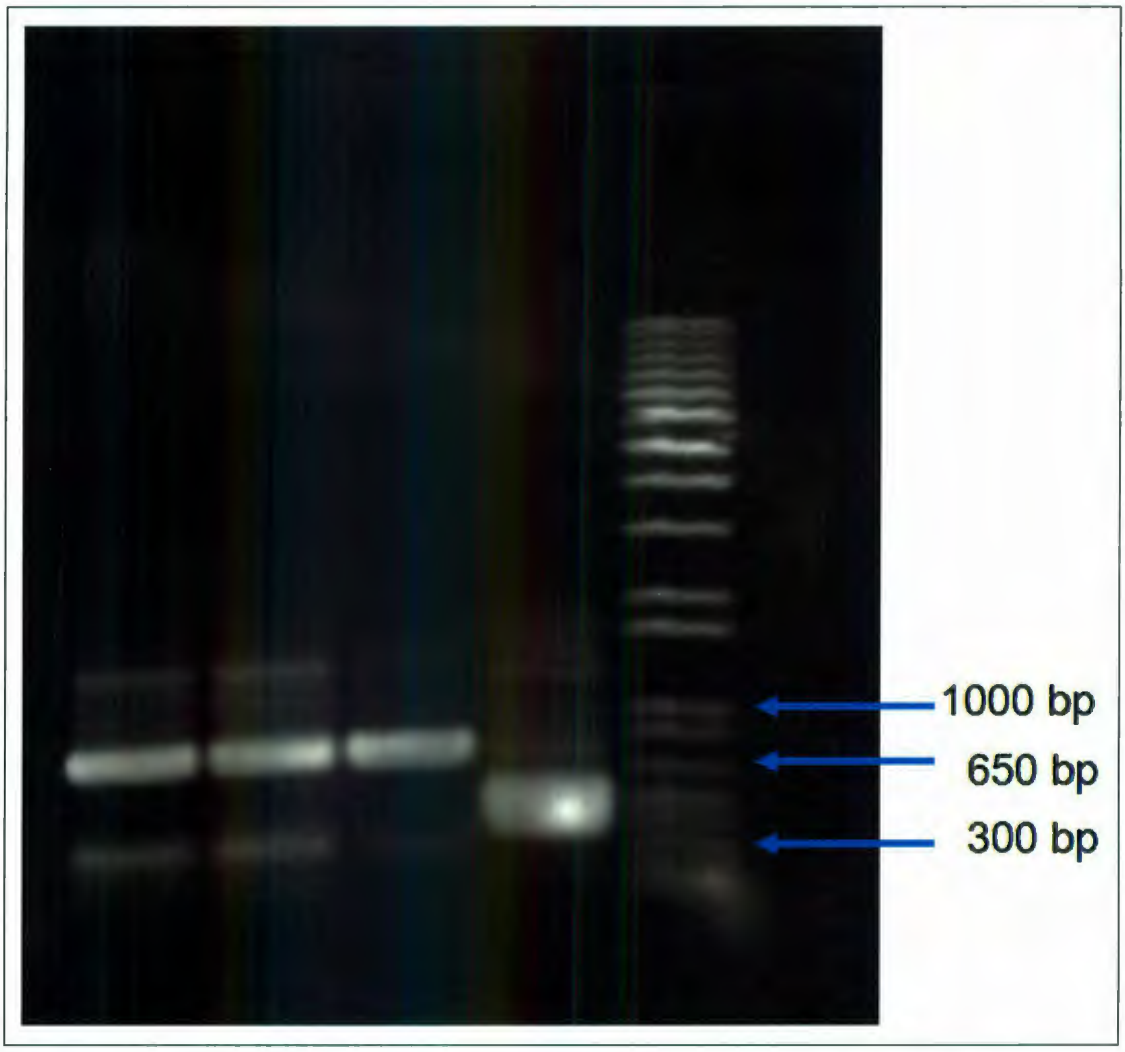

Figure 4.15 *dnmt6* splice block MO (3rd dnmt6 MO) was injected into embryos, and embryos collected at 72 hpf, RNA extracted, and RT-PCR performed. Expected nascent amplicon is 707 bp, and in the presence of MO, expected amplicon is 356 bp. Similar RT-PCR results were obtained for embryos collected at 12 hpf and 24 hpf. **Lane 1**: 10 ng *dnmt6* splice MO injected. **Lane 2**: 20 ng *dnmt6* splice MO injected. **Lane 3**: Danieau injected control embryos. Lower bands present in lanes 1 & 2 is also present, though fainter, in control sample. **Lane 4**: RT-PCR positive control (*max*). **Lane 5**: 1 kb Plus DNA ladder.

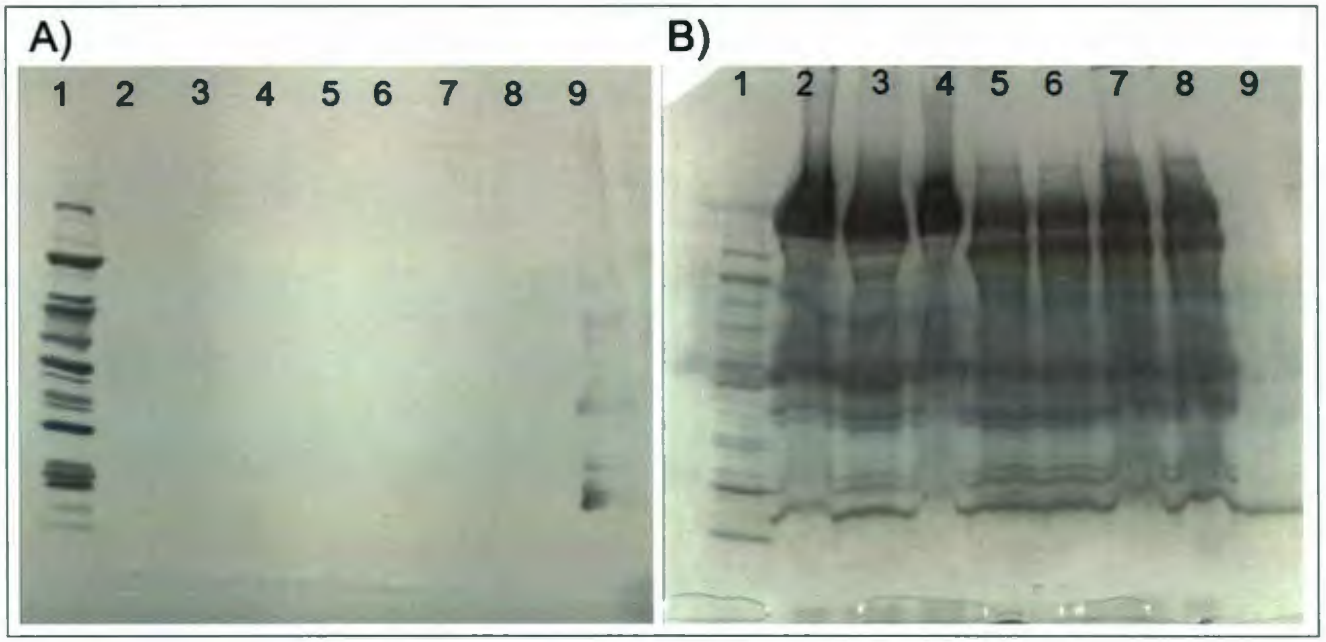

Figure 4.16 A) Western blot with protein extracted from embryos injected with His-tagged dnmt6 mRNA. **Lane 1**: Protein marker. **Lanes 2-4**: Embryos collected at 12 hpf. **Lanes 5-8**: Embryos collected at 24 hpf. **Lane 2**: Embryos received 0.76 ng dnmt6 His-tag mRNA. **Lane 3**: Embryos received 1.5 ng dnmt6 His-tag mRNA. **Lane 4**: danieau injected control embryos. **Lane 5-8**: Embyos injected with dnmt6 His-tag mRNA that was not modified to be refractive to the dnmt6 MO. This would indicate whether the modification at ATG start region was problematic in initial rescue experiments with modified ATG region. **Lane 9**: Positive control His-tag fragment. B) Coomassie Blue stained SDS gel prepared and loaded the same as the gel employed for western blot.

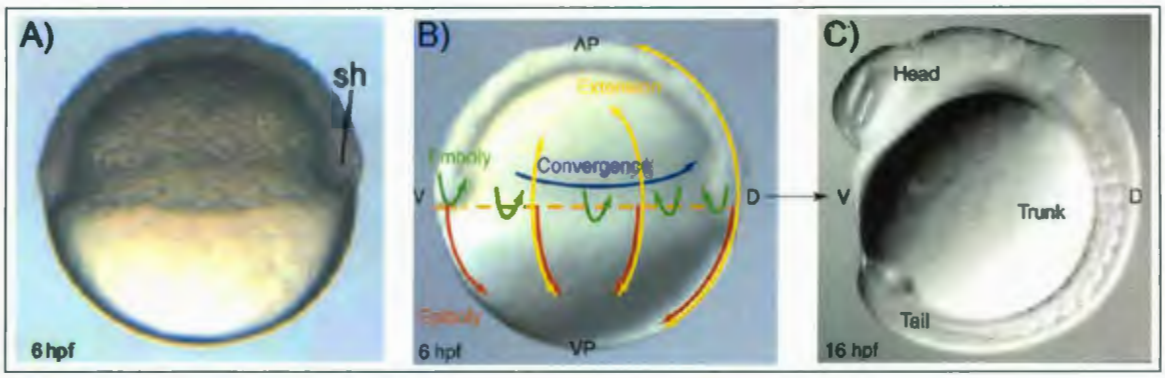

Figure 4.17 Gastrulation movements in zebrafish. A) Image of live embryo at shield stage and (B) the four gastrulation movements, epiboly, emboly (internalization), convergence and extension, that occur to transition the blastoderm into (C) an embryo with head, trunk and tail rudiments. The embryo elongates from head to tail, and narrows along the dorsoventral axis. Abbreviations: Sh, shield; AP, animal pole; D, dorsal; V, ventral; VP, vegetal pole (adapted from Solnica-Krezel 2006).

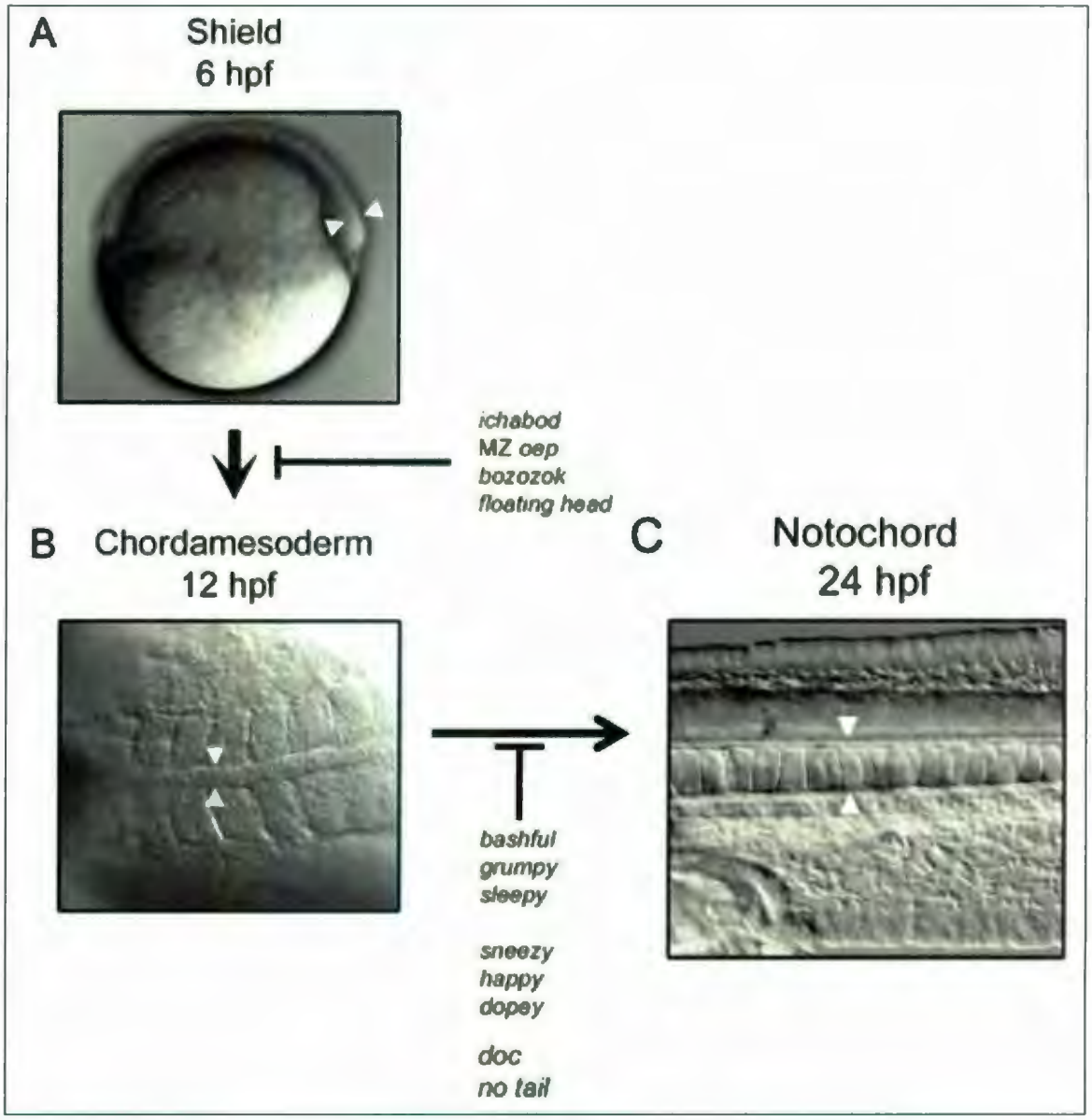

Figure 4.18 Notochord development and mutations that act at particular notochord developmental stages. A) The chordamesoderm progenitor region of the shield is indicated between arrow heads. B) The chordamesoderm and C) the differentiated notochord (with permission from Stemple 2005).

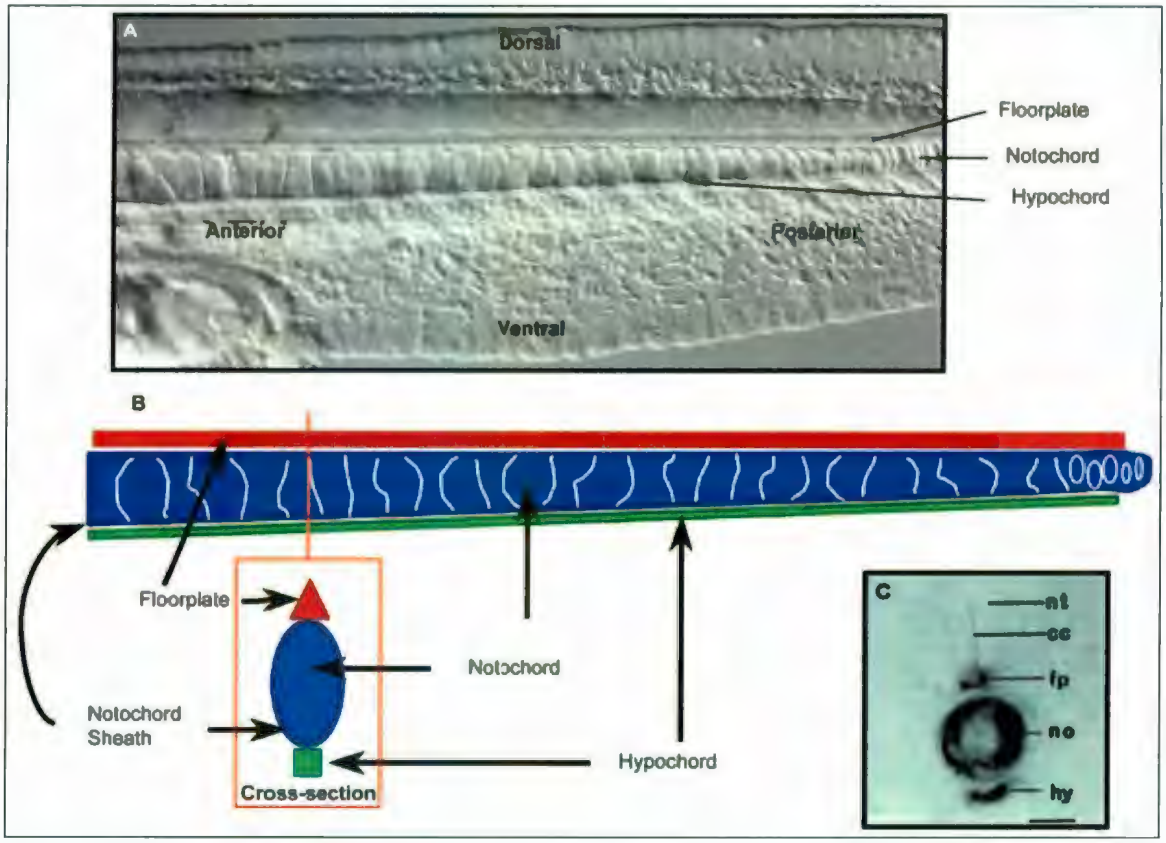

Figure 4.19 Structure of zebrafish notochord. A) Lateral view of a 24 hpf zebrafish tail showing the main features of the notochord. The floor plate is located dorsally to the notochord, in the ventral part of the forming spinal cord. The hypochord is located ventrally to the notochord. B) Schematic of lateral and cross-sections of the notochord. The floor plate and hypochord act as cables running along the top and bottom of the notochord. C) The notochord, the floor plate, and the hypochord express type II collagen (Yan *et al.*, 1995 from Stemple 2005). cc, central canal; fp, floor plate; hy, hypochord; no, notochord; nt, neural tube (with permission from Stemple 2005).

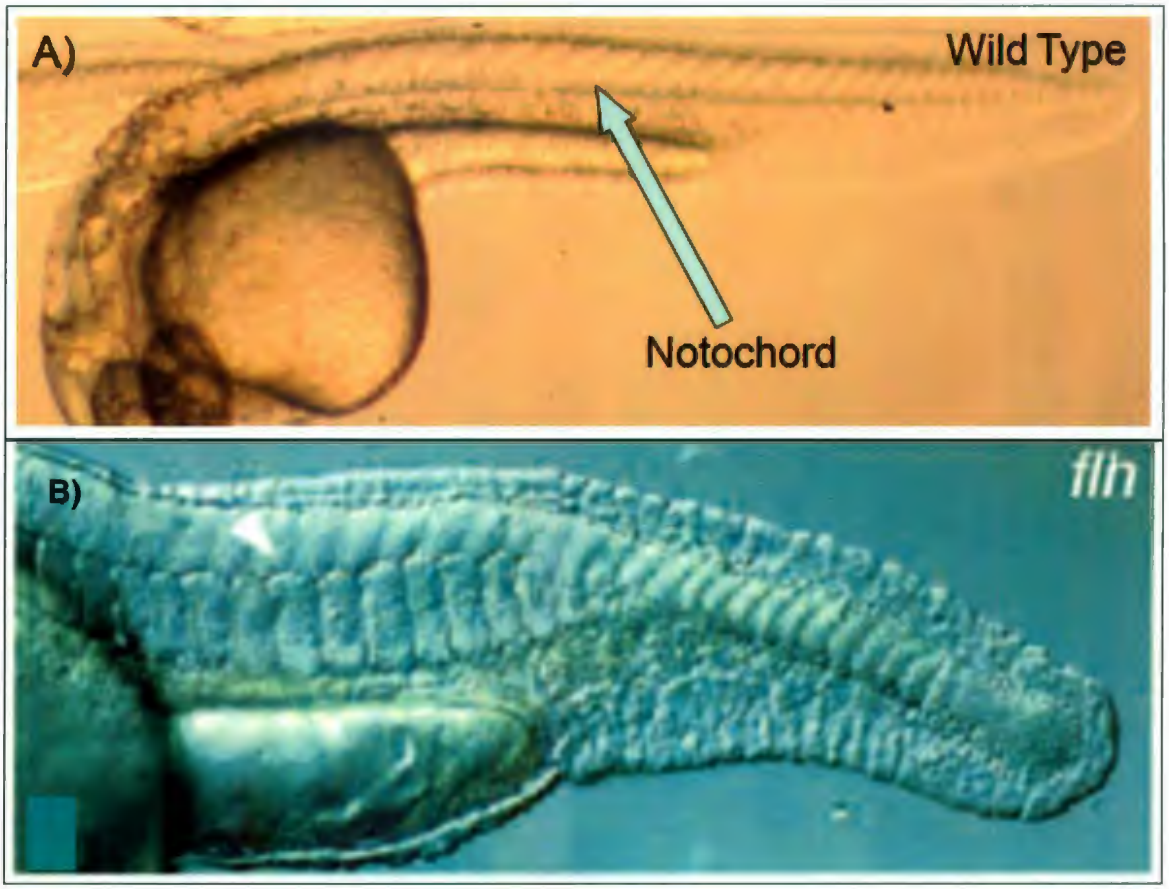

Figure 4.20 Live images of 24 hpf zebrafish embryos. A) wild-type embryo (arrow indicates notochord). B) *flh* mutant where chordamesoderm specification is disrupted (arrowhead indicates lack of notochord) (adapted from Odenthal *et al.* 1996).

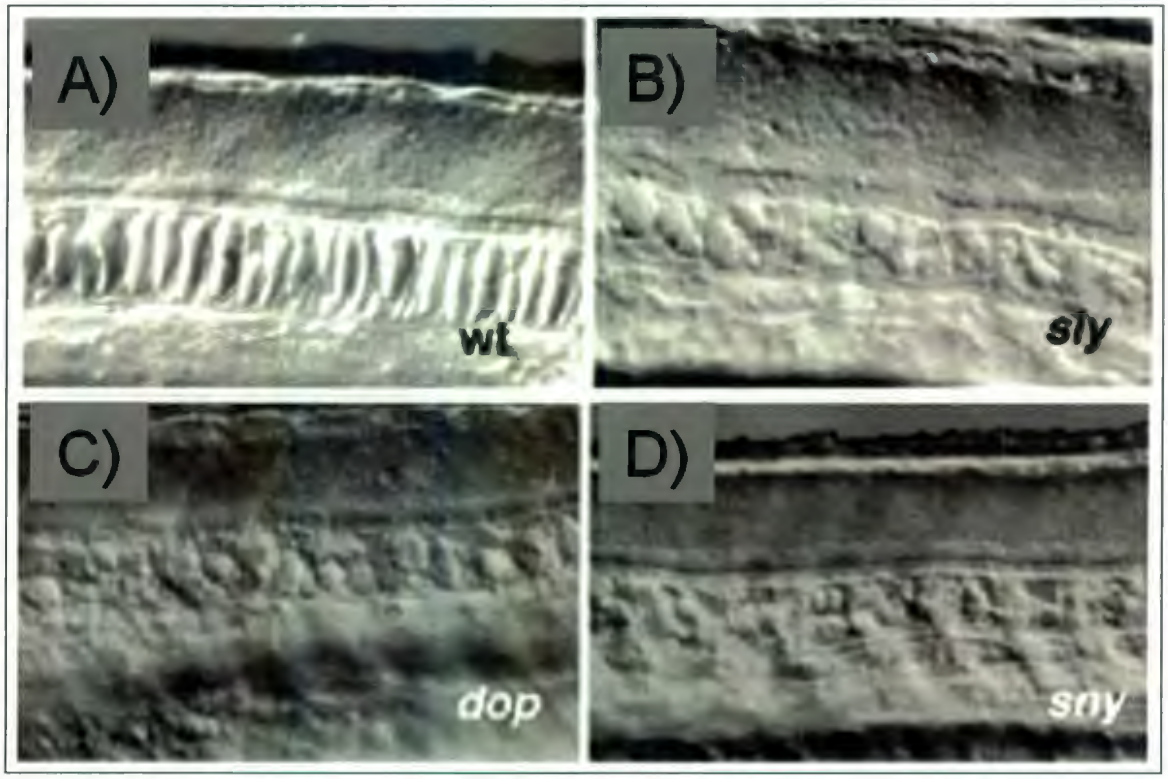

Figure 4.21 Differential interference contrast (DIC) micrographs of 24 hpf zebrafish embryos. A) wild-type embryo; B) *sleepy* mutants; C) *dopey* mutants; and D) *sneezy* mutants demonstrate notochord cells that fail to vacuolate. These loci encode components of the notochord membrane or components of the secretory pathway (with permission from Stemple *et al.* 1996).

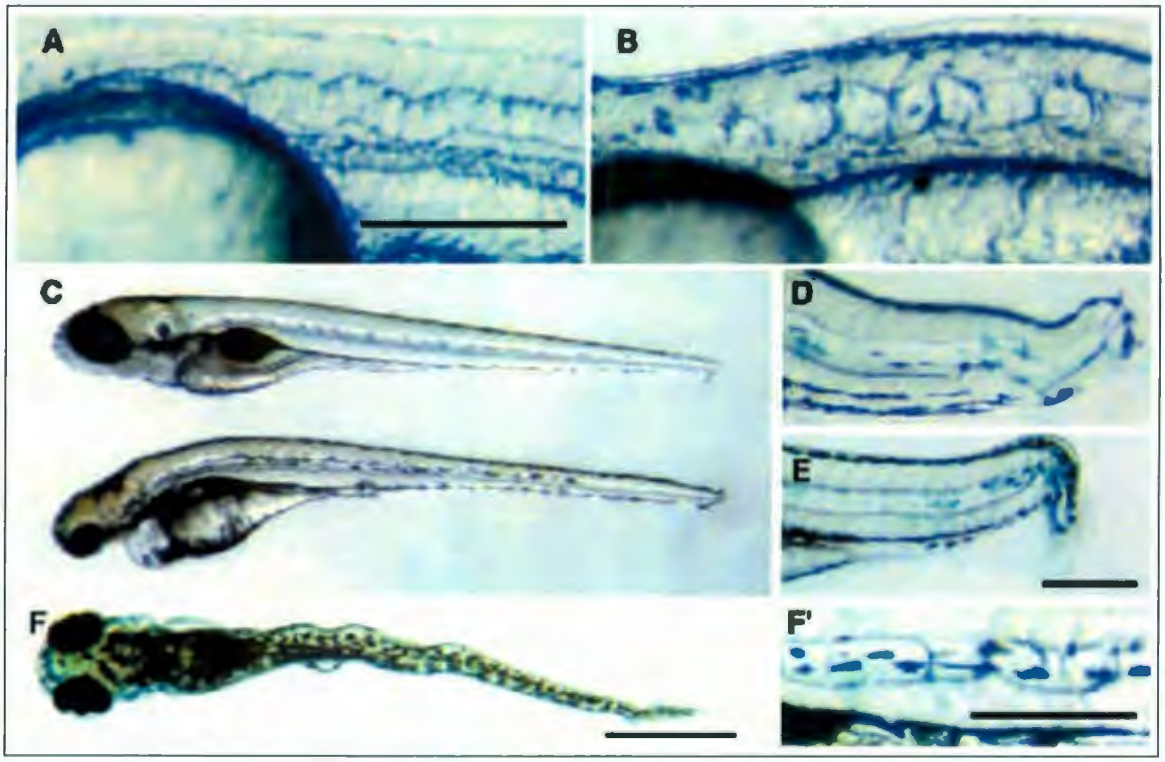

Figure 4.22 Images of zebrafish mutants *gullivar (gul), leviathan (lev), trilobite (tri),* and *knypek (kny).* A wavy notochord is apparent in A) *gul* and B) *lev* embryos by 28 hpf. C) By 72 hpf *gul* mutants notochord has straightened, though a severe head malformation is still apparent (bottom). F and F') By 96 hpf *lev* mutants, in contrast to *gul*, have an exacerbated notochord phenotype that shows both lateral (F) and dorsal-ventral (F') flexures. Gastrulation mutations also show folded notochord phenotype in D) *tri* and E) *kny* mutants at 72 hpf (with permission from Stemple 1996).

Table 4.1 Dose Response for *dnmt6* MO Injections and Control Injections.

Morpholino and Amount injected	Data	Total	Death Rate (%)	Embryos with Phenotype (%)
dnmt6 mismatch 5 ng	Sum of embryos with phenotype	0	47	0
	Sum of embryos injected	181		
	Sum of embryos survived	96		
dnmt6 mismatch 10 ng	Sum of embryos with phenotype	0	59	0
	Sum of embryos injected	98		
	Sum of embryos survived	40		
0 ng (Injection buffer + injection dye)	Sum of embryos with phenotype	0	25	0
	Sum of embryos injected	428		
	Sum of embryos survived	321		
dnmt6 MO 3 ng	Sum of embryos with phenotype	0	15	0
	Sum of embryos injected	95		
	Sum of embryos survived	81		
dnmt6 MO 5 ng	Sum of embryos with phenotype	14	22	19
	Sum of embryos injected	94		
	Sum of embryos survived	73		
dnmt6 MO 6 ng	Sum of embryos with phenotype	20	15	33
	Sum of embryos injected	71		
	Sum of embryos survived	60		
dnmt6 MO 7 ng	Sum of embryos with phenotype	77	10	35
	Sum of embryos injected	245		
	Sum of embryos survived	220		
dnmt6 MO 8 ng	Sum of embryos with phenotype	119	30	47
	Sum of embryos injected	360		
	Sum of embryos survived	252		
dnmt6 MO 10 ng	Sum of embryos with phenotype	43	37	45
	Sum of embryos injected	152		
	Sum of embryos survived	96		
0 ng Non-injected control embryos	Sum of embryos with phenotype	0	6	0
	Sum of embryos injected	266		
	Sum of embryos survived	249		

Table 4.2 dnmt6 MO and 2nd dnmt6 MO co-injections.

Mo Injected and Amount	*n* injected	% Phenotype (of survived)	% Death rate
5 ng *dnmt6* MO + 0.5 ng 2nd *dnmt6* MO	18	12 (2/16)	11 (2/18)
5 ng *dnmt6* MO + 1.0 ng 2nd *dnmt6* MO	18	15 (2/13)	27 (5/18)

Table 4.3 *dnmt6* MO and rescue *mRNA* injections.

Mo Amount	*n* injected	% Phenotype (of survived)	% Death rate	MO and rescue mRNA amount	*n* injected	% Phenotype with rescue (of survived)	% Death rate
10 ng	84	44 (16/36)	57	10ng MO + 0.5 ng mRNA	94	29 (16/55)	41

Chapter 5: Identification of Genes Affected by DNA Methylation Inhibition in Zebrafish Embryos: 5-aza-2'-deoxycytidine Treatment and Microarray Analysis

5.1 Introduction

The Azanucleoside drug 5-aza-2'-deoxycytidine (decitabine, 5azadC) functions as a DNA methylation inhibitor. This cytidine analogue contains a nitrogen atom in place of carbon-5 of the cytosine ring (Cisernos and Branch 2003). Following metabolic conversion into 5-aza-2'-deoxycytidine-5'-triphosphate, 5azadC may be incorporated into DNA by the replication machinery as a substitute to cytidine (Stresemann and Lyko 2008). Although DNA methyltransferases also recognize 5azadC as a natural substrate, once the methylation reaction is initiated, and the enzyme forms a covalent bond with carbon-6 of the cytosine ring, the reaction becomes blocked with the presence of the nitrogen atom in place of the carbon-5 atom. The enzyme remains covalently bound to the DNA, though incapable of carrying out methylation. As the functionality of DNA becomes compromised with the trapped methyltransferase, DNA damage signalling is initiated and the DNA methyltransferase is degraded (Stresemann and Lyko 2008). Therefore, methylation marks are lost not only due to inability of the methyltransferase to methylate 5azadC, but also through the degradation of the methyltransferases themselves.

Decitabine is currently used for its antileukemic activity in the treatment of myelodysplastic syndrome (MDS). Although the mechanism(s) responsible for remission in patients receiving decitabine remain controversial, one well-supported theory is that the promoter regions of tumour suppressor genes are hypermethylated in cancer tissue, and with decitabine treatment, hypomethylation results, leading to re-expression of genes relevant to differentiation, apoptosis, or senescence of the malignant clone (Griffiths and Gore 2008).

In addition, 5azadC has been employed both *in vitro* (Kharroubi *et al.* 2001; Enright *et al.* 2003; Hattori *et al.* 2004) and *in vivo* (Martin *et al.* 1999; Sasaki and Satoh 2007) to investigate normal DNA methylation function, particularly during development. A number of genes have been identified that are activated in the presence of 5azadC (Kharroubi *et al.* 2001; Hattori *et al.* 2004). Developmental abnormalities following treatment of embryos with 5azadC include delayed gastrulation and developmental arrest at neurula stages in *Ciona intestinalis* (Sasaki and Satoh 2007), and in zebrafish, disrupted gastrulation and aberrant patterning of dorsal mesoderm have been indicated (Martin *et al.* 1999). In mammals, DNA methylation has been demonstrated to be essential for development (Li *et al.* 1992), and when pregnant mice and rats are administered 5azadC, defects in pups include long bone defects, supernumerary ribs, cleft palate, and vertebral defects (Branch *et al.* 1999). Clearly, DNA methylation has a very critical role in early development, though there is very little knowledge as to the genes being affected to produce the above mentioned phenotypes. We present here an *in vivo* approach for 5azadC treatment, followed by microarray analysis, and importantly, in a vertebrate model of development, zebrafish, which demonstrate a similar global methylation pattern to mammals. Although some genes have been previously identified as being affected by methylation in zebrafish development through a candidate gene approach following 5azadC treatment (Martin *et al.* 1999), we hope to provide a more thorough examination of affected genes with more recently available zebrafish microarray technology.

5.2 Methods

Wild type zebrafish were raised at 28°C using standard methods (Westerfield 2000). Following fertilization, approximately 50 experimental embryos were raised in embryo medium treated with 5-aza-2-deoxycytidine (75 uM) (Sigma, St. Louis, MO) while approximately 50 control experiment embryos were raised in embryo medium. Optimal concentrations of 5-aza-2-deoxycytidine were previously determined (Martin *et al.* 1999), and confirmed here. Embryos were treated with 5azadC for 24 hours, following which embryos were collected as outlined below.

Approximately 40 embryos were collected per sample at 24 hours. Although staging is rendered difficult by delayed development and very often disrupted tail morphology upon 5azadC treatment, embryos with a heartbeat were determined to be 24 hpf. Total RNA was extracted from whole embryos using TRIzol reagent (Invitrogen Inc. Carlsbad, CA) with the manufacturer's instructions, genomic DNA contamination was removed with DNAse I treatment (Roche Ltd. Mississauga, ON), and this was followed with a final purification using the RNeasy clean-up kit (Qiagen Inc. Valencia, CA) with the manufacturer's instructions.

Aliquots of RNA were prepared for University Health Network (UHN) microarray center (Toronto, ON) and for quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) reactions. 5azadC RNA was Cy5-labelled and control RNA was Cy3-labelled (Quick Amp Labeling kit, Agilent Technologies. Mississauga, ON) with the manufacturer's instructions and gene expression changes in 5azadC treated embryos versus control embryos were determined by hybridization of labeled cRNAs to

Agilent 44 K zebrafish oligonucleotide microarrays (Hi-RPM Gene Expression Hybridization Kit, Agilent Technologies. Mississauga, ON). Three 44K zebrafish microarrays were hybridized with three independent 5azadC treated embryo samples and control embryos. Dye-swap experiments were not performed. The arrays were hybridized at 20 rpm for 17 h at 60 °C in a hybridization oven. Microarray analyses were performed at UHN microarray center. Arrays were scanned in the Agilent G2565BA DNA Scanner and quantified using Agilent Feature Extraction (version 9.5) and the array data was analyzed using GeneSpring (version 7.3.1. Agilent Technologies. Mississauga, ON). Features with intensities at or below average background levels were filtered out of the data set. Significant differences between 5azadC treated embryos and control untreated embryos were determined by signal fold differences of ≥2. These data have been deposited in NCBIs Gene expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/).

For gene validation following microarray analysis qRT-PCR was performed according to the manufacturer's protocol (LightCycler-2.0 Carousel Based System and LightCycler DNA Master SYBR Green I, Roche Ltd. Mississauga, ON). For RT reactions 1 ug RNA was reverse transcribed with M-MLV Reverse Transcriptase using Anc-T primer (Invitrogen Inc. Carlsbad, CA). qPCR reactions were performed using 2 ng cDNA and primers specific for the various genes (Table 5.1). Relative expression of genes in 5azadC treated embryos compared to untreated control embryos was determined by LightCycler Data Analysis (LightCycler Software 4.0) and Relative Expression Software Tool (REST) (Pfaffl *et al.* 2002). Gene expression of target genes was normalized to a non-regulated reference gene, elongation factor *elf4e1a* using REST

(Pfaffl *et al.* 2002). RNA used in reverse transcription for qRT-PCR was the same as that used for microarray experiments, and here, the three independently treated samples and their controls were examined in triplicate for each gene validation. Prior to determining relative expression, standard curves were determined and amplification efficiencies for the different primer sets were established. These values were used in calculating relative quantity PCR values. No template controls were also carried out alongside qRT-PCR reactions and dissociation curve analyses were performed.

Embryo fixation and DIG-labeled *in situ* hybridization were performed as described by Westerfield (2000). The RNA probe for the zebrafish *foxn4* corresponds to the following nucleotides: *foxn4* probe 998 nt (AF198446 nucleotides 443-1440. Forward primer 5'- CGGCCAGATTTCACTAGCTC, Reverse primer 5'- ACGTCAGTGTGGACCGTGTA).

5.3 Results and Discussion

To determine methyltransferase function in zebrafish development, we treated zebrafish embryos with 5azadC and found that the embryos displayed several developmental abnormalities. The most apparent disruption occurred to the notochord of the developing embryos. The tail and trunk length was often reduced, and in several instances, embryos lacked, in part or in whole, their notochord (Figures 5.1, 5.2 and 5.3) (also in Martin *et al.* 1999). In embryos where the notochord appeared to be mostly present, the notochord demonstrated a wavy or folded morphology. As well, somites and muscle underwent abnormal development (Martin *et al.* 1999). Another evident

phenotype that became more pronounced by 48 hpf was severe pericardial edema (Figures 5.2 and 5.3). Head size was often reduced, and very often eye development was affected. Embryos treated with 100 uM 5azadC demonstrated a higher death rate than lower doses, and therefore 75 uM treatments (death rate 15%) were used for treatments preceding microarray experiments. However embryos treated with the higher dose that did survive to 48 hours demonstrated a more severe phenotype (Figure 5.3). For a more complete analysis of phenotype resulting from 5azadC treatment of zebrafish embryos refer also to Martin *et al.* (1999).

Eighty-five genes were identified as being either induced or repressed by two-fold or more in embryos upon exposure to 75 uM 5azadC in all three repeated microarray experiments. If less stringent criteria are employed, such as 1.5-fold or greater change in at least two of the microarray experiments, 2347 genes with altered expression can be identified. A number of the genes with altered expression are known to be involved in differentiation, proliferation, CNS and skeletal muscle development. We verified a number of genes by qRT-PCR and found five of twelve genes examined are significantly induced or repressed in 5azadC exposed embryos relative to non-exposed control embryos by 1.5 fold or more (Table 5.1). The high false positive rate (58%) may be due to the fact that dye-swaps were not incorportated into the experimental design, and that only three replicates were performed.

Of the genes with significantly altered expression, transcription factor *fos* demonstrated the greatest increase in expression in response to 5azadC treatment compared to control embryos, with an average 8-fold increase. Fos is a component of the activator protein-1 (AP-1) transcription factor complex. When dimerized with members

of the Jun family to form the AP-1 complex, Fos is involved in gene regulation in processes of proliferation, differentiation, and transformation (Hess *et al.* 2004). Future investigations through bisulfite sequencing might reveal changes in DNA methylation that may be occurring during development in the promoter region of *fos*, or whether up-regulation seen here is due to mis-regulation of upstream genes.

In order to examine the biological significance of down-regulation of the winged helix/forkhead transcription factor, *foxn4*, we performed *in situ* hybridization on 5azadC treated embryos and control embryos. The Fox family of transcription factors in known to be involved in several aspects of development including cell specification, differentiation and proliferation (Cohen and Morrisey 2008). Specifically, *foxn4* has been demonstrated to have a role in formation of the atrioventricular canal, cardiac looping, and atrioventricular conduction (Chi *et al.* 2008; Cohen and Morrisey 2008) as well as retina and central nervous system development (Danilova *et al.* 2004). Expression of *foxn4* in normal zebrafish embryos has been previously characterized (Danilova *et al.* 2004) and demonstrates ubiquitous expression through to gastrula stages at which point tissue specificities begin to be observed. By 14 hpf, *foxn4* is expressed in three cell clusters in the forebrain region which will fuse and migrate anteriorly to form circular structures corresponding to the olfactory placode by 24 hpf. Expression is also apparent starting at 14 hpf in the anterior midbrain, and becomes restricted by 24 hpf to the periventricular layer of the tectum (Danilova *et al.* 2004) and around 20 hpf expression in the retina begins in the ventronasal region (Danilova *et al.* 2004). *In situ* analysis comparing normal zebrafish embryos (Figure 5.4) to 5azadC treated embryos demonstrated primarily a loss of expression in the olfactory placode and likely in the

retina (Figures 5.5 and 5.6). Some embryos also demonstrate loss of expression in the tectum, though this does not seem to be consistent across all treated embryos (Figure 5.6). This indicates that although qRT-PCR suggests a 3-fold decrease in expression of *foxn4* in 5azadC treated embryos, this down-regulation may reflect a range of disrupted expression *in vivo*.

vsx2 is another gene involved in retina development and proliferation. Embryos with disrupted vsx2 expression have reduced eye size (Vitorino *et al.* 2009). In 5azadC treated embryos we see a 2.7 fold decrease in expression at 24 hpf. Interestingly, *foxn4* has been shown to be down-regulated by *vsx2* in amacrine and horizontal progenitor cells of the retina. Vsx2, therefore, has been proposed to act as a gatekeeper functioning to repress transcription factors responsible for specifying progenitors of other lineages (Vitorino *et al.* 2009). Although in 5azadC treated embryos *vsx2* down-regulation is also accompanied by *foxn4* down-regulation, it will be interesting to discover the upstream genes whose mis-regulation results in down-reuglation of these genes and how they are involved in development of the severely disrupted retina phenotype.

5.4 Conclusion

Although in the past significant information has been gained through a candidate gene approach following 5azadC treatment, the more recent availability of zebrafish microarrays allow for the identification of a more comprehensive list of affected genes. The false positive rate for this microarray experiment is high, which suggests that it may be necessary to repeat this approach in the future with larger samples and greater number

of repeats in order to provide more robust information. In future investigations, embryos may be collected from various stages to identify not only the genes affected in various stages of development but may also identify general pathways that are affected.

Here we have identified a number of genes that appear to be affected by 5azadC treatment and, presumably, by decreases in methylation. Interestingly we have discovered a number of transcription factors with disrupted expression following 5azadC treatment. These factors have been shown to be relevant for different areas of development such as CNS and retinal differentiation and development. Future investigations may examine *in situ* expression to determine tissue specificities of the disruption, and more importantly, through bisulfite sequencing begin to examine precisely which genes are regulated developmentally by methylation, and provide target genes for examining further questions surrounding DNA methylation.

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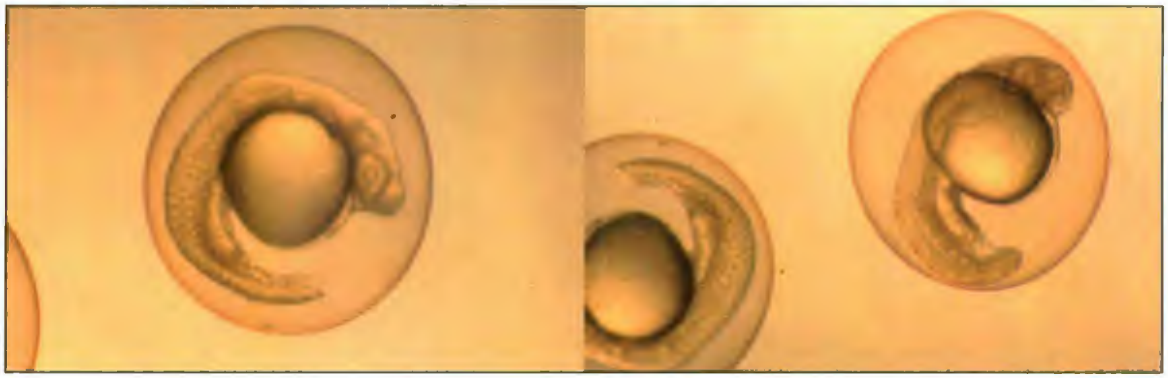

Figure 5.1 24 hour 75 uM 5azadC treated embryos. Disrupted tail and notochord development is evident.

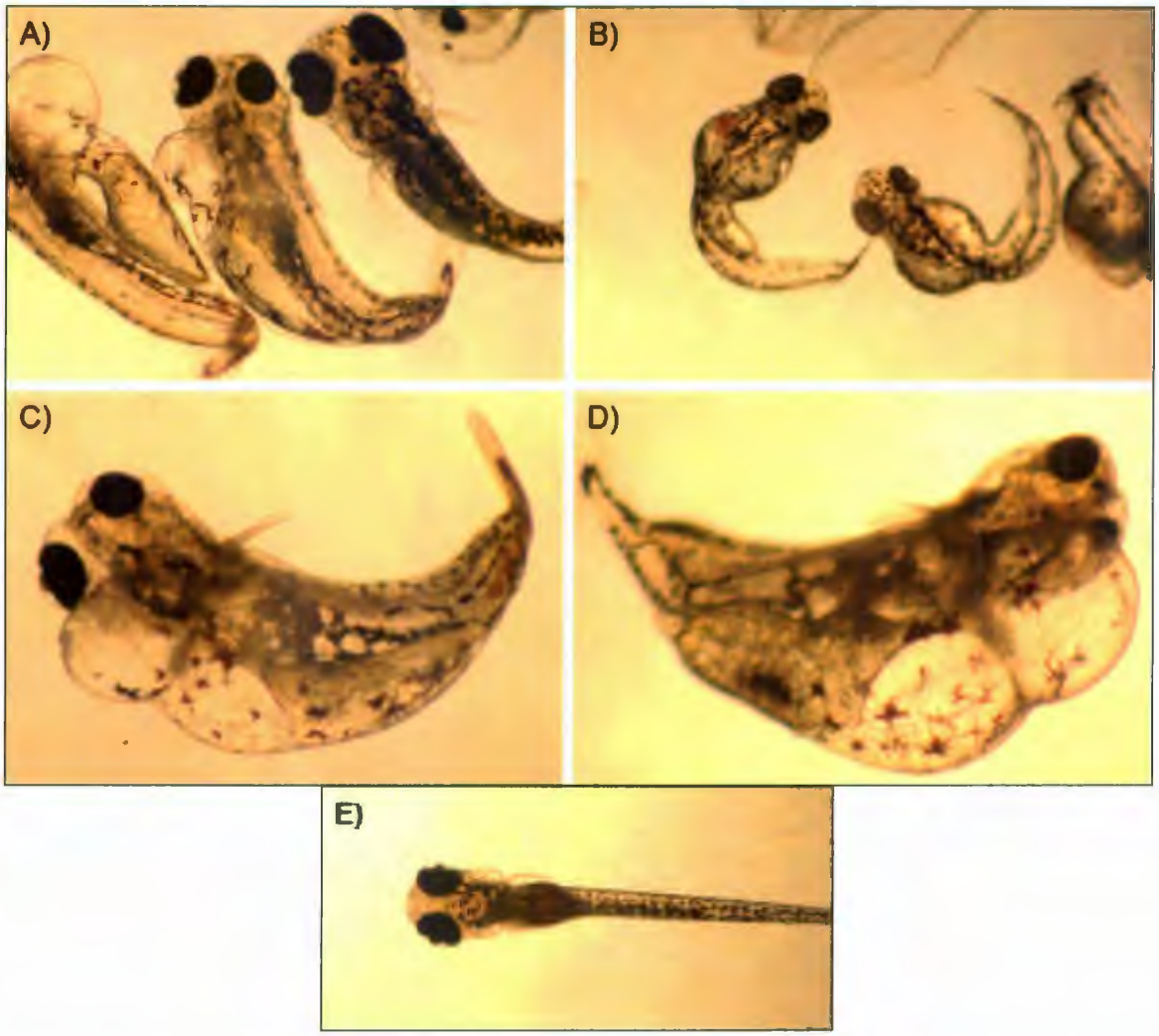

Figure 5.2 75 uM 5azadC treated embryos A & B) 4 days development C & D) 5 days development. Shortened trunk, disrupted notochord, and pericardial adema are all evident phenotypes. Additionally some abnormal eye development and blood pooling may be seen in some of the embryos. E) Control: 5-day embryo.

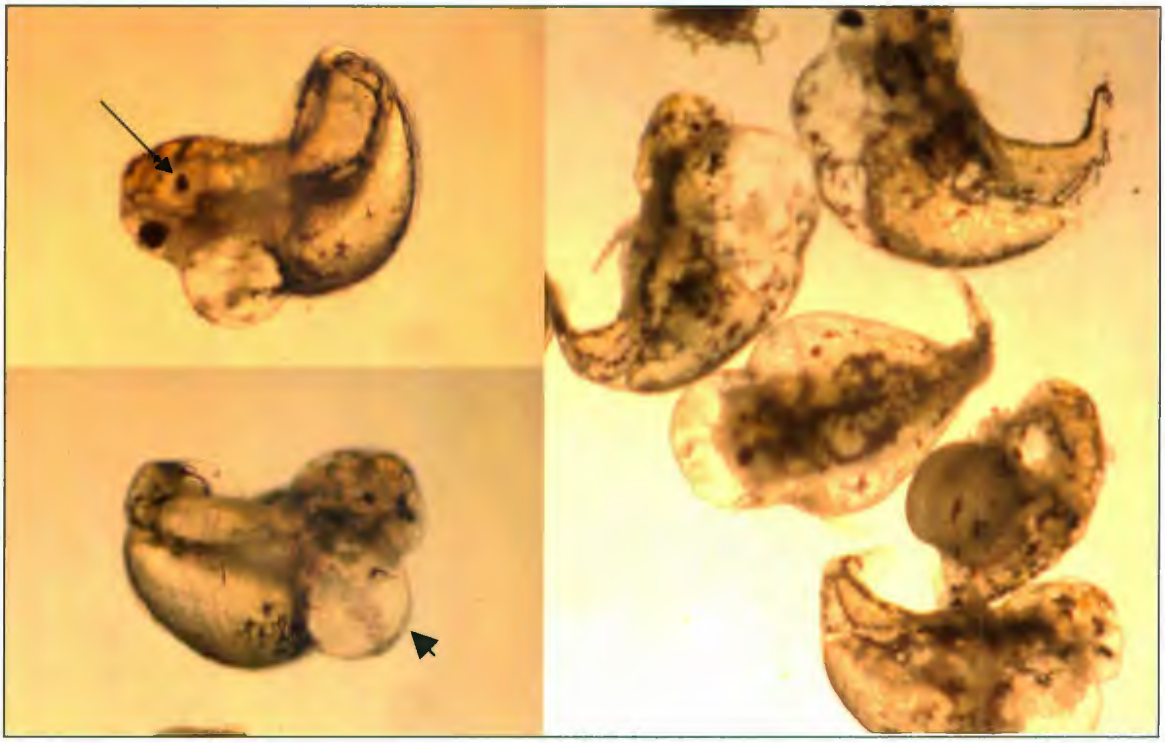

Figure 5.3 100 uM 5azadC treated embryos at 72 hours development. Very little eye development is observed in these embryos (arrow) and reduced trunk and tail is even more evident, as is reduced head size and pericardial adema (arrowhead).

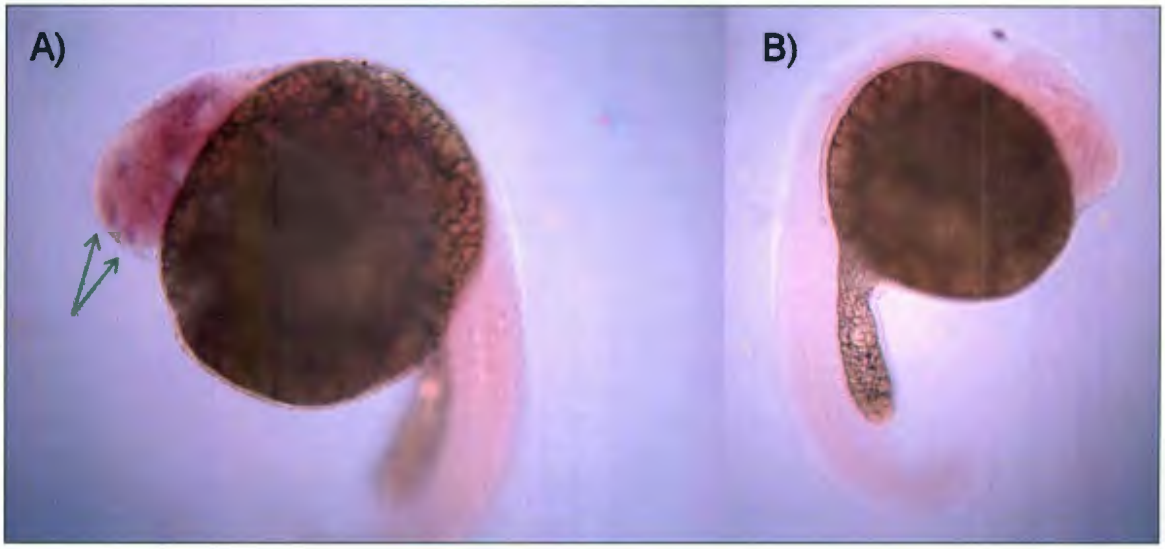

Figure 5.4 Wild-type 24 hour embryos. A) Untreated *foxn4* antisense embryos demonstrating normal expression. Green arrows indicate expression in olfactory placodes. Some staining is also evident in retina and tectum. B) Control *foxn4* sense probe shows no staining.

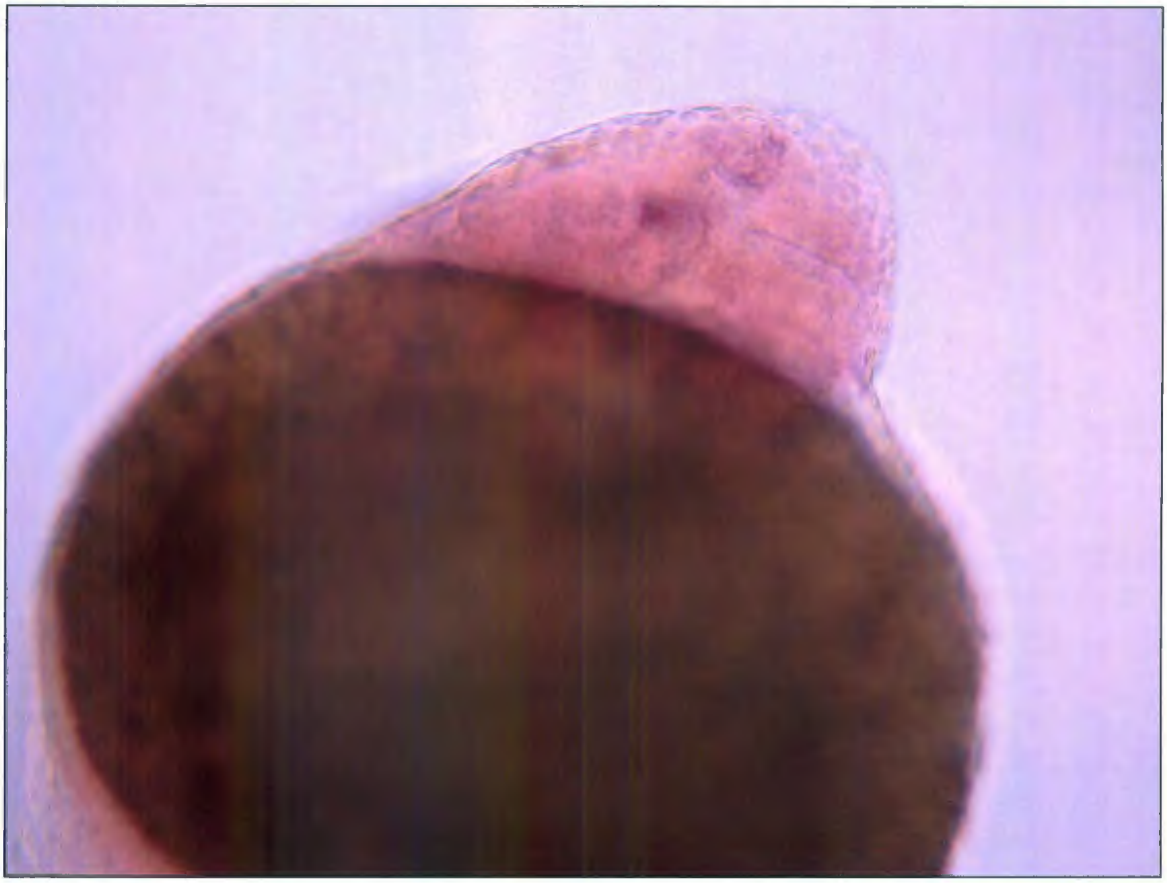

Figure 5.5 Wild-type 24 hour embryo treated with 75 uM 5zadC and *in situ* hybridization with *foxn4* antisense probe. Relative to untreated embryos, expression in olfactory placodes is not evident and at least reduced in retina and tectum.

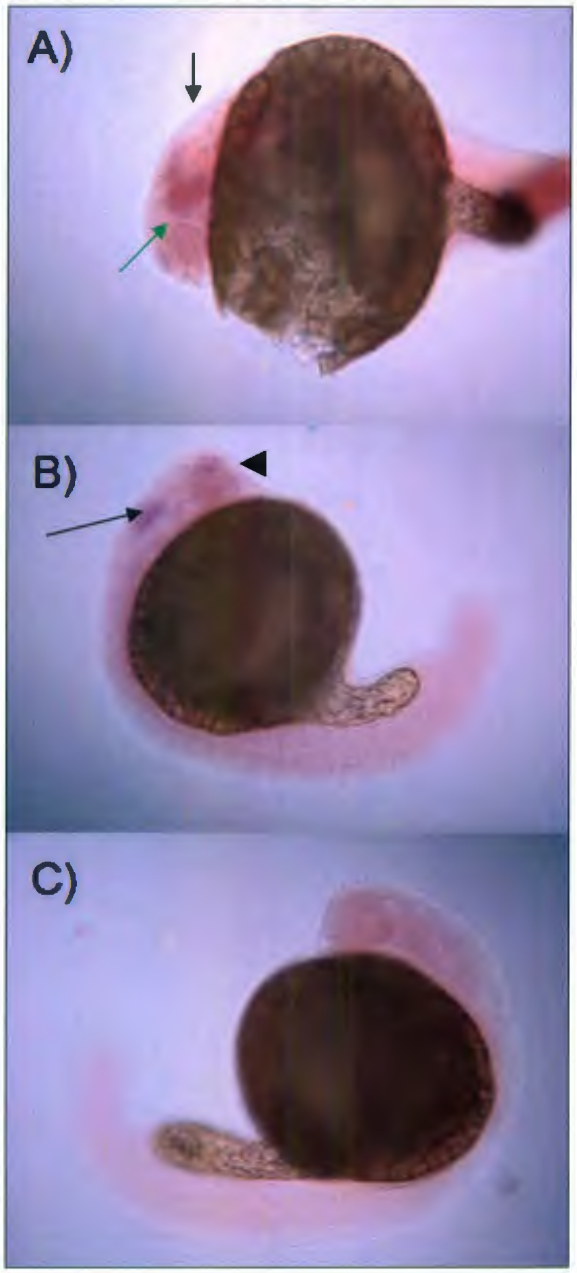

Figure 5.6 Wild-type 24 hour embryos treated with 75 uM 5zadC and *in situ* hybridization with A & B) *foxn4* antisense probe and C) *foxn4* sense probe. In A) no staining of olfactory placode (green arrow) is evident and reduced tectum staining (black arrow) is evident compared to B) where some cells continue to express *foxn4* in the tectum (black arrow) and retina (arrowhead). C) *foxn4* sense probe shows no staining.

Table 5.1 Genes examined by qRT-PCR for expression changes following 5azadC treatment. The factor of up- and down-regulation is indicated. For each sample three replicates were performed, and in instances where standard error is indicated, three replicates for three biological samples were performed. Genes that validated microarray results are highlighted (red = up-regulated, green= downregulated, yellow = non-regulated housekeeping gene).

Fold change (±*SE*)	Gene Name	Gene Description (accession number)	Primer for qRT-PCR (F=forward, R=reverse)
N/A	eif4e1a	eukaryotic translation initiation factor 4e 1a (NM_131733)	F 5'-GATGTCTGTGGTGCTGTCGT R 5'-TGTCCGCATGTGACTGGTAT
-0.81	vangl2	Danio rerio vang-like 2 (van gogh, Drosophila) (BC065983)	F 5'CAGGGGAATTGTGGGATATG R 5'CGCACCACTTTGAGACAGAA
-1.3	chd2	cadherin 2, neuronal (NM_131081)	F 5'-GTAACCTGCCCATGTCCAAC R 5'-AAACATCAACACCAGCACCA
-1.2	smarcc1	similar to SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily c, member 1 (BC047827)	F 5'TAAGGCGGAGTCAAAGGATG R 5'-CTTGGTCTCTCCTGCCTCAC
+1.16 (±*0.31*)	Icn	Ictacalcin (NM_212761)	F 5'-CCAGAAACATGAGTGGCAAA R 5'-CCCAAAGACATCGCTCATCT
+1.35 (±*0.31*)	slc6a11	Zebrafish WashU MPIMG EST Danio rerio cDNA clone IMAGE:3724908 (AI793569)	F 5'-CCCTGGCCTATGGTGAAGTA R 5'-GTTGATGGGAATGAGGGAGA
-1.07 (±*0.34*)	Provision-alck 739236	EA0189 Danio rerio one-day regenerating caudal fin subtracted cDNA Danio rerio cDNA clone 3-F04 (CK739236)	F 5'-AAGATCAAGCGAGCTTTTGC R 5'-ACCTGTCAAACGGTAACGCA
-1.18 (±*0.22*)	ribosomal pn S29	ribosomal protein S29 (TC279240)	F 5'-CGTCAGTGCTTCAGGCAGTA R 5'-CATGACGTCAGGTTGGACTG
+8.0 (±*4.66*)	fos	Finkel-Biskis-Jinkins (FBJ) murine osteosarcoma virus oncogene (NM_205569)	F 5'-AACTGTCACGGCGATCTCTT R 5'-TTGGAGGTCTTTGCTCCAGT
+1.6 (±*0.40*)	trim25l	RING+BBOX zinc finger protein (AY648763)	F 5'-AGAAGCTGAAGAGCCACGAG R 5'-CCTGTGTGGACACACTTTCG
-3.0 (±*0.20*)	foxn4	Transcription factor Foxn4, winged helix/forkhead (NM_131099)	F 5'-AGCTTTACTCCGCAAGACCA R 5'-CTTTTTGGCTGCCTTGTAGG
-2.7 (±*0.21*)	chx10; vsx2	visual system homeobox 2 protein (NM_131462)	F 5'-ACCAGTCTGCCAGCTCAGAT R 5'-CAGCATTTCTCTGGCGTACA
-1.5 (±*0.21*)	prox1	prospero-related homeobox gene 1 (NM_131405)	F 5'-TCCTTTCCCCCTTCCTCTAA R 5'-CTGGGTGTGTTGGTGAGATG

Chapter 6: General Conclusion

6.1 General Conclusion

As the study of epigenetics delves deeper, it is glaringly apparent that the level of complexity afforded by epigenetic mechanisms of gene control and the cross talk between these modifications gives rise to a system for which we are only beginning to scratch the surface in understanding. Implications in areas yet unknown are likely to continue to be uncovered.

Here we have demonstrated the power of using zebrafish embryos to ask a number of the questions surrounding DNA methylation in development. Initial steps involved identifying and isolating the *de novo* methyltransferases in zebrafish, of which there are six family members. The increased number relative to mammals is likely due to a genome duplication event, though in one instance we suggest simple tandem gene duplication as a possible origin of one of the genes rather than genome duplication.

Expression patterns of these genes in developing zebrafish embryos demonstrate similarities to patterns observed in mammals, suggesting conserved regulation in vertebrates. The presence of mRNA transcripts in early stages indicates maternal supply of each of the gene products. Following mid-blastula transition the *dnmt3*s are regulated independently demonstrating expression patterns that may be grouped based on whether the genes are more similar to mammalian *Dnmt3a* or *Dnmt3b*. Those zebrafish *dnmt3* genes most similar to *Dnmt3b* are more highly expressed relatively early in development, showing a decrease by 24 hours of development, suggestive of relevance through to gastrula and segmentation periods. In contrast, *dnmt8*, and in particular *dnmt6,* zebrafish *de novo* methyltransferases most similar to *Dmt3a,* demonstrate increasing expression

through to 72 hpf and therefore may be relevant for processes such as neurogenesis and terminal differentiation in addition to potential earlier role(s) in embryonic development.

Not only is the transcript expression pattern observed for *dnmt6* and *dnmt8* in development of particular interest, but also their prevelance in adult brain tissue. In recent years, research in DNA methylation in the CNS has seen a sudden increase given its implications in synaptic plasticity and long-term memory formation (Levenson and Sweatt 2005; Liu *et al.* 2007), as well as addiction (Hillemacher *et al.* 2009) and a number of mental health disorders such as Schizophrenia, and Fragile X mental retardation (Tsankova *et al.* 2007). In keeping with the idea that DNA methylation provides a mechanism for information storage at the cellular level during development, the emerging idea is that DNA methylation has been co-opted in the CNS for memory storage. DNA methylation in the CNS is dynamic, which also raises questions regarding the mechanism of active de-methylation. The CNS, in particular the brain, may provide a rewarding tissue therefore for investigating this highly sought yet undetermined mechanism.

In order to achieve our goal of determining the relevance of *de novo* DNA methyltransferases for different stages of normal development we injected MOs against *dnmt3, dnmt5, dnmt6, dnmt7,* and *dnmt8*. Interestingly, following MO injections targeting *dnmt6,* phenotypes that resulted from notochord malformation were observed. The phenotype is consistent with timing of expression of this gene; notochord specification and formation is coincident with the increase in *dnmt6* that commences at approximately six hours post-fertilization.

We did attempt some co-injections of MOs agains more than one *dnmt3* but, given that there are six *de novo* DNA methyltransferases, and a maintenance methyltransferase, knocking down greater numbers of *dnmt*s simultaneously by MO injection becomes problematic due to sheer number. Therefore, treatment of zebrafish embryos with 5-azadC provided a way of interfering with all methylation and, consequently, giving a more comprehensive understanding of the overall importance of DNA methylation to zebrafish development. This technique accompanied with microarray analysis provided insight into genes that are either directly or indirectly regulated by methylation.

One very obvious similarity between the *dnmt6* MO knockdown and the 5azadC treatment was the effect of both on notochord development and differentiation suggesting that DNA methylation is very relevant to those processes. As the notochord phenotype was more severe in embryos following 5azadC treatment than in *dnmt6* MO knockdown embryos, it is likely that other DNA methyltransferases are also involved in notochord formation and/or differentiation. Very possibly, based on timing of notochord specification, and determined expression, some of the earlier expressed dnmt3s may be involved in specification and early formation while dnmt6 (and possibly dnmt8) are involved in differentiation. This would be consistent with areas of absence of notochord in 5azadC treated embryos, versus an undulating notochord in dnmt6 knockdown embryos. Similar findings of a transition from Dnmt3b to Dnmt3a have been indicated by expression analyses in the development of the CNS in mice (Feng *et al.* 2005).

Specifically, we determined through microarray analysis a number of genes affected by 5azadC that are involved in differentiation including *foxn4*, *fos*, and *vsx2*. Some of these genes may also therefore be relevant to the phenotypes observed in *dnmt6* MO

knockdown experiments as they have been implicated in heart and eye development. Although we did not determine a number of genes as expected to be directly involved with notochord differentiation, it is possible that examination of an earlier developmental stage may provide further insight.

Overall, we have demonstrated through the identification of genes homologous to mammalian *de novo* methyltransferase genes, and by identifying similarities observed in their transcript expression, that zebrafish embryos are a very favourable system to pursue analyses of DNA methylation. We have undertaken investigations into the function of DNA methylatransferases in zebrafish development and demonstrated that methylation is critical to notochord development, and is also likely relevant to proper somite, retina, and heart development as evidenced by phenotypes observed following morpholino injection and/or chemical treatment of embryos with dnmt activity blocking 5-aza-2'-deoxycytidine. Furthermore we have begun to identify genes relevant to the abovementioned phenotypes that are directly or indirectly regulated by methylation. This has been achieved through microarray analysis of 5azadC treated embryos.

Given these advances, along with the strengths afforded by zebrafish for this area of research such as rapid ontogeny and observable live embryos throughout development, the presence of epigenetic factors, lack of imprinting, and global methylation patterns, this model organism is highly suited to further investigations of DNA methylation and epigenetics. Results attained here suggest future investigations with zebrafish embryos to be of great impact in our understanding of epigenetic mechanisms of gene control.

6.2 Future Directions

Future investigations should continue to determine presence of other splice variants for the zebrafish *dnmt3* genes, for which there is some preliminary evidence from RT-PCR reactions. In addition, probes of greater nucleotide number may be employed to increase specificity in *in situ* transcript expression analysis, and more comprehensive *in situ* analysis of embryos and adult tissues such as brain will provide further detailed information on the regulation and relevance of the different *dnmt3* genes to zebrafish development.

The main focus, however, should be to co-inject problematic MOs, discussed in chapter 4, with a p53 MO, in tandem with continued development of morpholinos that efficiently and specifically knockdown the various zebrafish *de novo* methyltransferases. Most importantly, these MOs should be used in combination with one-another in order to determine functional overlap among this family of genes and very likely, when homologous dnmts are knocked down simultaneously, developmental phenotypes will be observed and may be studied. One might expect these phenotypes to build up to the very severe phenotype observed when overall methylation is disrupted by 5azadC treatment.

Preliminary investigations may be carried out by examining genes indicated by 5azadC treatments in MO injected embryos by both real-time PCR and/or *in situ* hybridization to determine which of the methyltransferases is relevant to the expression of the identified genes. However, microarray analysis following the various MO injections will provide specific and comprehensive insight into the genes being regulated developmentally by the various methyltransferases. Microarray experiments may also be

performed to examine different stages of development in order to determine the timing of methylation events and indicate the various processes affected. Furthermore bisulfite sequencing of some of the genes, and heterochromatic regions may be carried out to determine whether there are significant methylation changes detectable in either form of chromatin. These genes, once they have been determined to be affected, will mark the starting point for studying promoter methylation changes, dnmt3 targeting, and interactions between epigenetic layers.

These suggested investigations of DNA methylation and its role in notochord specification and differentiation will be of special interest given the many unanswered questions surrounding neural tube defects and in particular the role of DNA methylation in these defects. Intriguingly, following 5azadC treatment, zebrafish embryos exhibit a shortened body axis, a phenotype commonly associated with convergence extension defects (Solnica-Krezel *et al.* 1996; Weiser *et al.* 2009). Many neural tube defects have been attributed to abberant convergence extension, with the molecular disruptions and causes of those disruptions still under investigation (Kibar *et al.* 2007). These connections, in addition to the use of folic acid (a critical factor in the synthesis of the methyl group carrier SAM) to prevent neural tube defects (Taparia *et al.* 2007), certainly warrants pursuing 5azadC treated embryos at earlier stages to investigate whether in fact convergent extension is affected. These investigations of DNA methylation in morphogenesis would represent a novel area of research.

6.3 Literature Cited

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